MODULATION OF SENSORY FUNCTION BY HIGH GLUCOSE-INDUCED OXIDATIVE STRESS: ROLE OF TRPV1 RECEPTORS

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ABSTRACT

Diabetes can have a devastating effect on sensory nerves often leading to clinical symptoms of pain abnormalities termed diabetic sensory neuropathy (DSN). Converging evidence demonstrate that oxidative stress plays a pivotal role in DSN as a result of elevated reactive oxygen species (ROS) induced by hyperglycemia and lead to sensory neuron injury. However, the cellular and molecular mechanisms initiating neuronal dysfunction in DSN are still poorly understood. The present thesis investigated the effects of high glucose-induced oxidative stress on sensory function mediated by the activation of purinergic (P2X) and vanilloid (TRPV1) receptors expressed in nociceptive neurons from neonatal mouse dorsal root ganglion (DRG). Primary cultures of lumbar DRG neurons were prepared to characterize sensory function during control (5 mM glucose) and high glucose (25 mM glucose) conditions using electrophysiological, live imaging and biochemical techniques. Interesting, while characterizing TRPV1 channels following repetitive applications of capsaicin, I noticed two distinct responses in TRPV1 activity: a 'monophasic' response, commonly observed as a run-down of peak currents; and a 'biphasic' response characterized as a gradual potentiation followed by a run-down of peak currents. The latter response is a novel behaviour that had not been characterized under control condition, and this prompted further investigations. I revealed that the gradual potentiation was independent of calcium entry, and was mediated by PKCβ. Under the high glucose condition, the two responses remained unaffected; however, I observed an enhancement of TRPV1-mediated currents, while P2X-mediated currents seemed to be unaffected. Furthermore, I demonstrated that the enhancement of current can be prevented with the use of antioxidants. This suggests that the enhancement of TRPV1 activity by high glucose- induced oxidative stress may play an essential role in peripheral sensitization in diabetes.

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AGE	advanced glycation end-product
ALA	α-lipoic acid
ASO	antisense oligonucleotide
AP	action potential
ATP	adenosine triphosphate
Ba^{2+}	barium
CAP	capsaicin
CAT	catalase
Ca^{2+}	calcium ion
$[Ca^{2+}]_i$	intracellular calcium concentration
CGRP	calcitonin gene-related peptide
DH	dorsal horn
DMSO	dimethyl sulfoxide
DRG	dorsal root ganglion
DSN	diabetic sensory neuropathy
ECF	extracellular fluid
EGTA	ethylene glycol-bis(ß-aminoethyl ester)- N,N,N',N'-tetracetic acid
HEPES	N-2-hydroxyethylpiperazine-N'-2-etheansulfonic acid
HG	high glucose
H_2O_2	hydrogen peroxide
HS	horse serum
IGF	insulin-like growth factors
L15	leibovitz L-15 medium
mEPSC	mini excitatory postsynaptic current
Na^+	sodium ions
NADH	nicotinamide adenine dinucleotide hydride
nAChR	nicotinic acetylcholine receptor
PKC	protein kinase C
P2X	puringeric receptor

P2X2	homodimeric purinergic receptor subtype 2
P2X3	homodimeric purinergic receptor subtype 3
P2X2/3	heterodimeric purinergic receptor subtype 2 and 3
ROS	reactive oxygen species
STZ	streptozotocin
SP	substance P
TRPA1	transient receptor potential ankyrin 1
TRPV1	transient receptor potential vanilloid 1
TRPV3	transient receptor potential vanilloid 3

CHAPTER 1

GENERAL INTRODUCTION

Diabetes, a disease characterized by chronic elevation of plasma glucose, results from either insufficient insulin production in type 1 diabetes, or from insulin resistance in type 2 diabetes. If left unchecked, this elevated glucose, or hyperglycemia, damages the cardiovascular system, the retina, the kidney, and the peripheral nerves. As a result, many diabetics are predisposed to life-threatening myocardial infarctions and stroke, and develop blindness, kidney failure and experience uncontrolled pain or the loss of sensation in the lower limbs (Vinik et al., 2003a; Calcutt et al. 2009). While these system-wide abnormalities can seriously affect the quality of life and shorten the life expectancy of diabetic patients, some of these conditions have been shown to be a greater burden on the patient and health care system both medically and economically. Affecting nearly 50% of the diabetic population, the clinical syndrome referred to as diabetic neuropathy can inflict multiple symptoms reflecting sensory, motor, and autonomic nerve dysfunction, depending on the peripheral nerve affected by this pathology. However, a common condition is the display of sensory abnormalities characterized by incapacitating pain or loss of protective sensation in the lower limbs, and thus the predisposition to injuries leading to poor wound healing, foot ulcerations and gangrene. If untreated, the end result is lower extremity amputation (Boulton et al., 2004). The severity of diabetic neuropathy has prompted considerable research, much of it focused on glucose metabolism and metabolic pathways that lead to glucose toxicity in neurons and resulting in neuronal damage in diabetic neuropathy (reviewed by Tomlinson & Gardiner, 2008). However, the cellular and molecular mechanisms that are affected by glucose neurotoxicity and contributing to the manifestations of these symptoms remain poorly understood. In this thesis, I am studying mechanisms that affect sensory neurons from the dorsal root ganglion (DRG), neurons that are relevant in pain processing and contribute to the deleterious sensory abnormalities of diabetes, to help in the search for new therapeutic strategies to treat this debilitating condition.

1.1 Sensory neurons of the Dorsal Root Ganglion (DRG)

1.1.1 Origin and differentiation of DRG neurons

The dorsal root ganglia are a part of the peripheral nervous system that transduces afferent sensory information from the external world or the internal environment of the body into electrochemical signals that the central nervous system then processes. The DRG which is comprised of a heterogeneous population of sensory neurons that detect a variety of sensory modalities, originates from the neural crest in the developing embryo. The neural crest is found at the border of the ectoderm and the neural plate, where folding of the neural plate initiates the migration of multipotent neural crest cells (NCC) that will differentiate into sensory neurons of the DRG (reviewed by Marmigère & Ernfors, 2007). The different lineages of sensory neurons are determined by the presence of neuronal determination genes, such as neurogenin 1 (Ngn1) or 2 (Ngn2) in multipotent NCC that expresses Sox10, an important transcription factor in sensory neuron lineage (Carney et al., 2006) that has demonstrated its principal role in generating different classes of sensory neuron (Ma et al., 1999). The migration of NCC away from the neural crest occurs in three waves of neurogenesis (Marmigère & Ernfors, 2007). In the first wave, the expression of Ngn2 has been shown to commit NCCs into cells that express the neurotrophin receptors tyrosine kinase B (TrkB) and/ or C (TrkC). The second wave of neurogenesis involves the proliferation of Sox10-expressing cells that may differentiate into glial cells or facilitate the third wave of neurogensis, in which the expression of Ngn1 commits the proliferated Sox10-expressing NCCs into a population of DRG neurons containing the transcription factor Runx1 and TrkA.

Runx1 has been shown to be an important transcription factor in differentiating peptidergic DRG neurons, which express calcitonin gene-related peptide (CGRP) and substance P (SP), from nonpeptidergic DRG neurons during postnatal development. Gene manipulation studies revealed that Runx1 induces changes in neurotrophic factor dependency in DRG neurons. Neurons expressing TrkA, the receptor for neurotrophic growth factor (NGF), switch from TrkA expression to the expression of Ret, a receptor for the glial-derived neurotrophic factor (GDNF), in response to Runx1 (Chen et al., 2006). This transition was accompanied by the repression of CGRP (Kramer et al., 2006) in DRG neurons, suggesting that Runx1 was critical in segregating nonpeptidergic DRG neurons from peptidergic neurons. In addition, behavioural analysis of null

Runx1 mice revealed deficits in thermal and neuropathic pain (Chen et al., 2006), suggesting the involvement of this transcription factor in the expression and/ or function of nociceptors in sensory neurons of the DRG during development.

1.1.2 Anatomy and Classification of DRG neurons

The DRG are nodule-like structures situated near the ventrolateral surface of the vertebra1 column of the spine. Each ganglion is a collection of cell bodies of sensory neurons. Sensory neurons are classified as pseudo-unipolar, and are characterized by a single axonal projection from the distal end of the neuronal soma that traverses for a short distance before bifurcating. The bifurcation of the axon sends one axonal projection, identified as the central terminal, to the dorsal horn of the spinal cord and the second axonal projection, identified as the proximal terminal, is sent to the skin, visceral organs, muscles or joints (Fig. 1-1, Liu & Ma, 2011).

Because there are a number of tactile sensory modalities (i.e. temperature, nociception, proprioception, pressure, vibration and itch), the ability to identify a specific modality has been attributed to the distribution of a heterogeneous population of sensory neurons. One classification scheme is based on the morphological characteristics of DRG neurons: (1) large cell bodies (diameter > 35 μm) are typically associated with myelinated Aβ fibers and transmit information on proprioception and vibration; and (2) small (diameter < 25 μm) to medium (diameter >25 and <35 μm) cell bodies are typically associated with unmyelinated C fibers or thinly myelinated Aδ fibers that are responsible for conducting information on nociceptive (thermal or mechanical) stimuli (Lawson 1979 & 2002). Another classification scheme is based on the expression of neuropeptides, i.e. CGRP and SP. This classification scheme allows for further segregation of small DRG neurons into peptidergic and nonpeptidergic neurons (Averill et al., 1995; Molliver et al., 1995; Molliver et al., 1997).

Various types of sensory neurons characterized above are affected during the pathological conditions generated by diabetes. However, small to medium peptidergic DRG neurons have been reported to be more vulnerable to diabetes-induced cellular injury and to be critical components for the manifestation of diabetic sensory neuropathy (Adeghate, Rashed, Rajbandari & Singh, 2006; Johnson, Ryals & Wright, 2008).

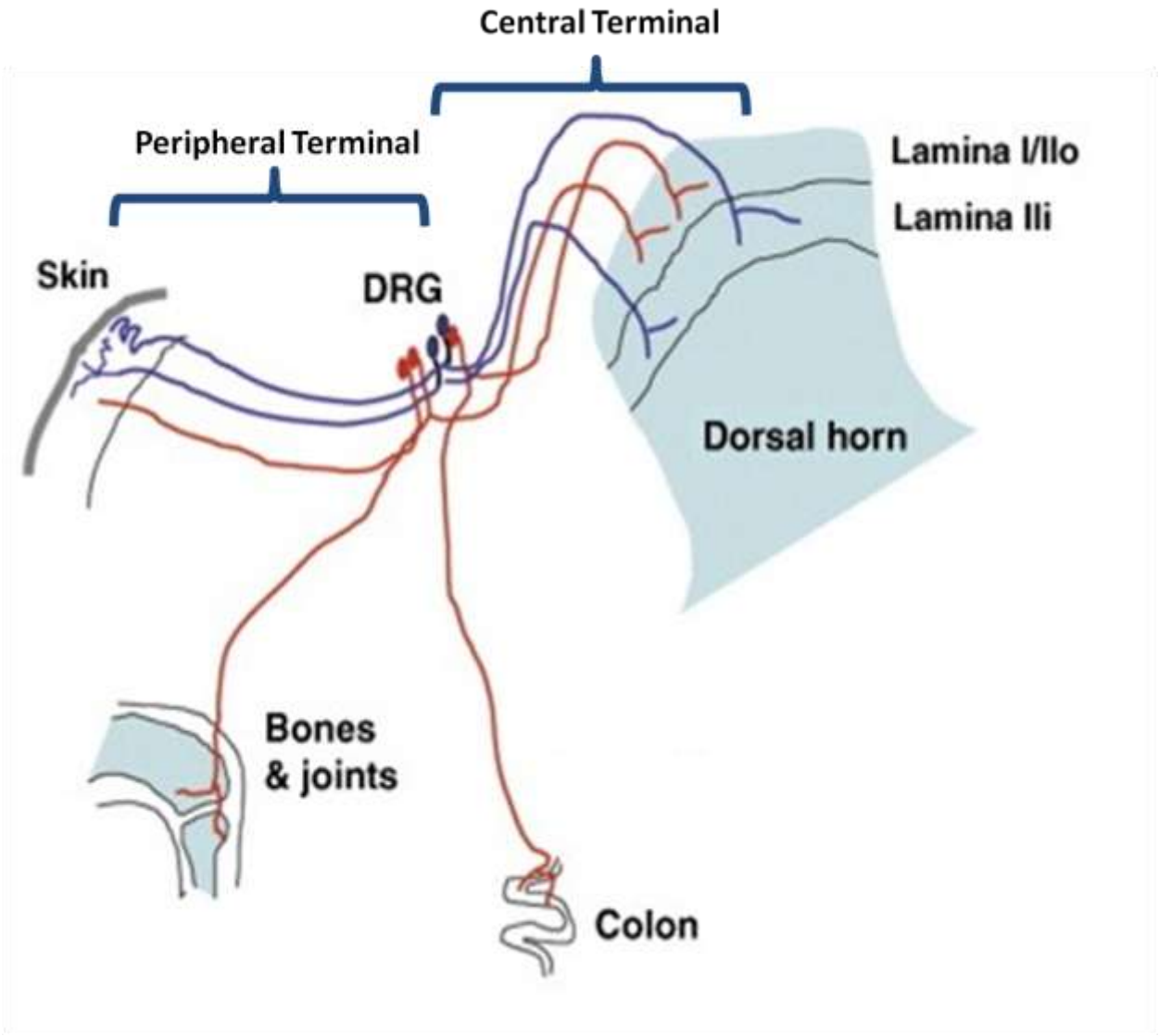

Figure 1-1. **Differential innervations of the DRG.** Sensory neurons of the dorsal root gangliong (DRG) receive sensory information from the external world (i.e. skin) and internal environment of the body (i.e. bones and joints, tissues and organs) via the peripheral axon terminals of the DRG. The transduced electrical signals from the soma of the DRG neurons are then relayed to the dorsal horn of the spinal cord via the central axon terminals of the neuron. **(Figure modified from Liu & Ma, 2011)**

1.2 Diabetic sensory neuropathy

Diabetic neuropathy is the most common health complication associated with diabetes, and reflects peripheral nerve dysfunction and/or damage (Vinik et al., 2003a). A vast majority of diabetics display signs of sensory abnormalities that start at the feet and ascend proximally, which is referred to as diabetic sensory neuropathy (DSN). Sensory abnormalities in DSN are described as incapacitative pain with or without sensory stimulation or the lack of sensation in the lower limbs that predisposes the patients to injuries leading to poor wound healing, foot ulcerations and sequelae of events with an end result of limb amputation (Boulton, 2004; Guastella & Mick, 2007). In most cases, the severe pain state (i.e. neuropathic pain) in the lower limbs precedes the loss of sensation and is related to long-term diabetes and nerve damage. However, in other cases, DSN patients do not exhibit the typical neuropathic pain but rather the loss of sensation in the same lower extremity (reviewed by Vinik, 2003b). Clinical studies have highlighted two independent processes that may be associated to the differences in the type of sensory abnormality: the degree of nerve fiber regeneration and the degree of axonal atrophy. It was reported that the rate of successful nerve regeneration was greatest in diabetic patients exhibiting pain abnormalities in DSN, and the degree of axonal atrophy was greatest among diabetic patients exhibiting the loss of sensation in DSN (Behse et al., 1977; Britland et al., 1990). However, it remains unknown what factors contribute to the differential sensory abnormalities observed in DSN patients.

Because the degree and magnitude of the painful sensory abnormality in DSN can vary across patients, a greater emphasis in the literature has been on understanding the mechanisms regulating the neuropathic pain state in DSN. The incapacitative pain in diabetic patients has been described as a burning, throbbing or shooting sensation that can occur spontaneously, chronically or intermittently (Dworkin, 2002). Characterization of the type of pain states have included: hyperalgesia, severe pain towards a stimulus that evokes a slight painful sensation, or allodynia, severe pain in response to an innocuous stimulus that normally does not induce pain. A noninvasive diagnostic aid to evaluate sensory abnormalities was made possible by the use of a dermatome map. Sensory examination (i.e. vibration, thermal, and mechanical stimulation) of a dermatome, an area of the skin that sends afferent information to a single spinal nerve of the DRG, would identify sensory abnormalities localized to specific DRGs along the spinal cord. The abnormal sensation characteristic of DSN (i.e. neuropathic pain or lack of sensation) is

predominantly localized to the lower limbs (i.e. leg, foot and toes) and is shown to correspond to DRGs of the lumbar segments of the spinal cord. The susceptibility of the lumbar spinal nerves of the DRG to the pathogenesis of DSN is thought to be due to its long axonal projection from the spinal cord segment to the distal limbs. Lumbar DRGs have therefore been a prime candidate in examining cellular and molecular mechanisms affected by DSN.

Another diagnostic instrument commonly used is the direct examination of skin biopsies looking at the sural nerve that terminates at the epidermis on the lateral side of the ankle and foot. Sural nerve biopsies have been used as a valid tool to examine the progression of nerve fiber loss in diseases and in treatment-based regeneration and functioning of the nerve fibers (Pringle et al., 1974; Griffin et al., 2001). Clinical investigation into early pathological changes in the sural nerves biopsy from diabetic patients (diagnosed with diabetes mellitus for more than 6 months) revealed an early occurrence of small unmyelinated fiber degeneration, measured as a decrease in axon diameter, without any changes in nerve conductance or velocity, but with a compensatory increase in the unmyelinated axon density and Schwann cell profile (Malik et al., 2005). In contrast, clinical assessment in patients with long term diabetes revealed extensive loss of myelinated and unmyelinated fibers with a concomitant decrease in axon diameter, reduction in sensory nerve conduction velocity associated with a decrease in myelination, and reduced Schwann cell profiles that indicated a reduced capacity for nerve regeneration (Bertelsmann et al., 1985; Llewelyn et al., 1991; Malik et al., 2001; Polydefkis et al., 2004). Because of the susceptibility of small unmyelinated fibers to diabetes, it has been suggested that the functional and structural changes to these fibers were responsible for the severe pain exhibited by diabetic patients.

Unfortunately, there are no treatments for DSN other than glycemic control and diligent foot care (Rathur et al., 2005; Sullivan & Feldman, 2005; Little et al., 2007; Vinik et al., 2003a & 2004). This is in spite of ongoing research addressing the pathogenesis of the disease, with the goal to identify mechanism-based treatments. In particular, the influence of glucose-mediated pathways in the pathogenesis of DSN involved in the functional changes in sensory neurons. In recent years, research in animal models of diabetes have suggested that dysregulation of glucose levels activates multiple distinct metabolic pathways that lead to a singular end result: oxidative stress in neurons (see below).

1.3 Oxidative stress in diabetes

It is well established that hyperglycemia mediates oxidative stress through multiple mechanisms resulting in the overproduction of reactive oxygen species (ROS). Four glucose mediated pathways have been implicated in the generation of ROS by increased: (1) polyol pathway flux; (2) formation of advanced glycation end-products (AGE); (3) activation of protein kinase C (PKC) isoforms; and (4) hexosamine pathway flux (reviewed by Brownlee, 2001). The increase in metabolic flux within these pathways has reportedly influenced the electron transport chain of the mitochondria. The increase in reducing agents, such as NADH, coupled with the depletion of ATP and increase in electron availability resulted in the formation of ROS at the proximal portion of the electron transport chain (Vincent et al., 2002; Tomlinson & Gardiner, 2008). The inability of the cell's antioxidant defense system to compete with the excessive amount of ROS rendered the cell vulnerable to oxidative damage, and subsequently altering the structure and function of lipids, DNA and protein, which ultimately led to programmed cell death. Other oxidative stress responses have also been suggested to influence ROS production. For example, loss of neurotrophic support by insulin and the insulin-like growth factors (IGF-I, IGF-II), nerve growth factor and neurotrophin-3 (Chiarelli et al., 2000), as well as altered intracellular calcium homeostasis and calcium signalling (Huang et al., 2002; Fernyhough & Calcutt, 2010). Although the cellular mechanisms elicited by ROS remain unclear, it is well established that hyperglycemia leads to oxidative stress.

The effect of oxidative stress has been studied in cell cultures of DRG neurons as a simplified model to explore the underlying mechanisms in DSN. Small to medium sized dissociated DRG neurons exposed to a hyperglycemic insult resulted in mitochondrial membrane potential dysregulation, mitochondrial swelling and the overproduction of ROS (Russell et al., 2002; Vincent et al., 2002 & 2005a). Exposure to mild concentrations of pro-oxidant agents (e.g. H_2O_2) initiated of a transient defense mechanism augmenting the expression of antioxidant enzymes to counteract ROS production (Vincent et al., 2009). However, this defense mechanism has been reported to be compromised in DRG neurons exposed to higher concentrations of glucose (Vincent et al., 2009).

The influence of the glucose-mediated pathways and the accumulation of ROS have led to a few hypotheses to explain the role of oxidative stress in peripheral nerve malfunction in diabetes. It was initially hypothesized that the accumulation of cytoplasmic ROS led to the initiation of multiple effector mechanisms that triggered apoptosis or programmed cell death in DRG neurons (Russell et al., 1999; Vincent et al., 2002; 2005a; 2009). These studies showed a decrease in the number of DRG neurons as early as 2 hours following exposure to high glucose. This finding was consistent with previous studies that reported apoptosis in endothelial cells involved in micro- and macrovascular injuries leading to retinopathy and nephropathy (Obrosova et al., 2003 & 2005). Unfortunately, the level of hyperglycemia used in these studies was greater than those observed in diabetes, which makes the validity and reliability of these findings to DSN questionable. A more recent hypothesis for the development of DSN is based on the synaptic malfunction of peripheral nerves due to axonal swelling and retraction, and the inability of axonal regeneration (Srinivasan, 2000; Fernyhough & Calcutt, 2010). Rather than oxidative stress-induced apoptosis, Fernyhough and colleagues (2010) reported that elevation of ROS triggered mitochondrial dysfunction, which causes axonal dystrophy, but spares neuronal cell bodies. The inability to relay sensory information from the DRG to the dorsal horn neurons of the spinal cord seems to be a plausible hypothesis for DSN, although mechanisms resulting from elevated cytoplasmic ROS and contributing to functional and structural changes in DRG neurons remain poorly understood.

More recently, oxidative stress has been directly implicated in the onset of diabetic autonomic neuropathy. Autonomic neuropathy is a form of diabetic complication that affects autonomic nerves causing autonomic malfunction or dysautonomia. Campanucci et al. (2008 & 2010) have elucidated the mechanisms underlying the onset of dysautonomia in animal models of type 1 and type 2 diabetes. Hyperglycemia-induced elevation in cytoplasmic ROS caused the inactivation of neuronal nicotinic receptors (nAChRs) in sympathetic neurons by targeting conserved cysteine residues located near the intracellular mouth of the nAChR channel. Because nAChR "drive" sympathetic synaptic transmission, their inactivation leads to depression in ganglionic transmission and reduced sympathetic reflexes. Importantly, the use of antioxidants and neurons expressing mutated nAChRs subunits that lacked the conserved cysteine residues, prevented the inactivation induced by hyperglycemia and animals showed normal sympathetic

reflexes. These findings proposed a functional (rather than structural) abnormality in sympathetic ganglia induced by diabetes, opening a new window of opportunity for therapeutic strategies in the treatment of diabetic neuropathy. Whether cytoplasmic ROS have any effect on receptors expressed in the DRG neuron remains unclear.

1.4 Neurotransmitter receptors in DRG neurons involved in nociception

Nociceptive DRG neurons are comprised of a heterogeneous population of neurotransmitter receptors. For example purinergic (P2X) receptors and transient receptor potential 1 (TRPV1) have been identified as main players in sensory function, in particular, of nociception (Chen et al., 1995; Caterina et al., 1997). Although these receptors have been extensively studied with regards to pain processing, their function during hyperglycemic conditions and their possible contribution to DSN remains elusive.

1.4.1 Role of P2X receptors in nociception and diabetes

P2X receptors are membrane ion channels activated by the binding of extracellular adenosine triphosphate (ATP). They belong to a large family of ion channels widely expressed in the nervous system and play an important role in many physiological and pathological processes (Grubb & Evans, 1999). The DRG neurons express a variety of P2X receptors (Chen et al., 1995; Lewis et al., 1995). To date, seven different subclasses have been reported (P2X1-7; North, 2002). The highest proportion of mRNA transcript expressed in DRG neurons are the homodimeric P2X3 receptor, followed by P2X2 receptors and the heterodimeric P2X2/3 receptors (Chen et al., 1995; Lewis et al., 1995; Stebbing et al., 1998). Notably, these 3 subclasses are distinct in their phenotypic responses: (1) fast-inactivating currents have been associated with P2X3 receptors; (2) slow-inactivating currents associated with P2X2 receptors; and (3) mixed response of fast and slow inactivated currents were expressed in P2X2/3 receptors (Grubb & Evans, 1999). Remarkably, *in vitro* studies have shown that greater than 80% of DRG neurons expressed fast-inactivating currents reflecting P2X3 receptors (Grubb & Evans, 1999).

Studies from genetic models in which the expression of P2X3 receptors was manipulated revealed their critical role in processing of thermal and mechanical nociception (reviewed by Jarvis, 2003). Deletion of P2X3 receptors caused a loss of the rapidly desensitizing ATP-evoked

currents that paralleled normal responses to noxious mechanical and thermal stimuli and reduced formalin-induced pain behaviour (Souslova et al., 2000). In addition, down regulation of P2X3 receptors by antisense oligonucleotide treatment decreased P2X3-mediated currents (Dorn et al., 2001) and reduced mechanical hyperalgesia (Barclay et al., 2002) in models of inflammation and chronic pain.

Because P2X3 is the predominant purinergic receptor subtype expressed in DRG neurons, its role in pain is relatively understood. However, understanding of the role of P2X2 and P2X2/3 receptors remains limited as a result of their scarcity in the literature of pain. A significant contribution was provided by Cockayne et al (2005), who studied primary cell culture of DRG neurons from null mice lacking the P2X2 subunit or from a double null mice lacking the P2X2 and P2X3 subunits. The study revealed that sensory neurons from P2X2 null mice responded to ATP with only transient inward currents, while neurons from P2X2/P2X3 double null mice had minimal to no response to ATP. These data indicated that P2X receptors on sensory ganglion neurons involved almost exclusively P2X2 and P2X3 subunits. Consistently, both P2X2 null and P2X2/P2X3 double null mice had reduced pain-related behaviours in response to intraplantar injection of formalin, suggesting that these P2X subtypes may not be crucial to nociceptive processing.

Only recently have the roles of P2X receptor subunits in animal models of diabetes been investigated. In diabetic animal models, in which the insulin-producing β cells of the pancreas are destroyed by streptozotocin (STZ; Schnedl, Ferber, Johnson & Newgard, 1994), there was an increase in expression and function of purinergic receptors in mice (Migita et al., 2009) and rats (Gy, Li, Liu & Huang, 2011). Migita et al (2009) reported an increase in P2X2 and P2X3 mRNAs at 5 days following STZ injection, which remained stable at 14 days. This increase in mRNA transcripts paralleled the onset and sustained hyperalgesic response during von Frey filaments test, a test for mechanical nociception. More recently, Gy et al. (2011) examined P2X-mediated currents from DRG neurons following 2 weeks of STZ-induced diabetes in rats. The study revealed ATP-evoked currents that resembled the P2X3 subtype and observed a two-fold increase in inward current versus control. This potentiation paralleled the increase in P2X3 expression in the plasma membrane of DRG neurons, while total expression of the P2X3 receptors remained unaltered. To supplement their findings, behaviour analysis using the von

Frey test also revealed mechanical hypersensitivity that was alleviated by nonspecific P2X receptor antagonist PPADS. Due to the low specificity of the antagonist, it is unknown whether P2X2 or P2X2/3 receptors were contributors to diabetes-induced mechanical hyperalgesia. Nevertheless, these studies indicate that P2X receptors are influenced under diabetic conditions.

Due to recent evidence on the influence of oxidative stress in diabetes, the affects of oxidative stress on the purinergic receptor has also been described in cultured HEK 239 cells (Mason et al., 2004; Coddou et al., 2009). Mason et al. (2004) reported selected modulation of purinergic receptors as a result of hypoxia-induced ROS production. Under acute hypoxic conditions, P2X2 receptors had shown a significant decrease in current amplitude compared to cells in normoxic conditions. In addition, modulation of ROS production via the mitochondrial electron transport chain altered the current magnitude in P2X2 receptors. A decrease in ROS production via the inhibition of mitochondrial complexes 1 and 3 caused a transient increase in ATP-induced current, while an increase in ROS production via inhibition of complex 3 caused a transient decrease in current magnitude. Although Mason et al. (2004) described the influence of ROS on P2X2 receptors, the exact mechanism by which ROS altered the structure and/or function of the ion channel remains unknown. Moreover, the authors reported that this modulation observed in P2X2 receptors had no effect on homodimeric P2X3 and heterodimeric P2X2/3 receptors. Contrary to these findings reported by Mason et al. (2004), a recent study by Coddou et al. (2009) reported a potentiation in current magnitude for P2X2 receptors in cells in response to H_2O_2, a type of ROS. The authors described this potentiation as a result of oxidization of intracellular cysteine residues. When cells were exposed to reducing agents, ROS-mediated potentiation of current magnitude was prevented. However, the involvement of this mechanism in the context of diabetes remains poorly understood. Interestingly, these *in vitro* studies implicate P2X2 receptors as the sole target of acute oxidative stress, contrasting the effect seen in diabetic animal models in which both P2X2 and P2X3 were implicated.

1.4.2 Role of TRPV1 receptors in nociception and diabetes

In addition to P2X receptors, the TRPV1 receptor has been extensively studied in pain processing and may also be influenced by DSN. Ablation of TRPV1 led to deficient responses to noxious (thermal or mechanical) stimuli (Caterina et al., 2000; Mishra et al., 2011), suggesting a pivotal role for TRPV1 in the detection and processing of pain. In addition, pharmacological

manipulations of the receptor demonstrated that the TRPV1 receptor is a polymodal detector activated by a number of stimuli: capsaicin, protons (pH), heat (>42°C), and arachidonic acid (Caterina et al., 1997). TRPV1 is a nonselective cation channel that depolarizes the membrane potential of neurons due to its high permeability to calcium ions (Wood et al., 1988; Docherty et al., 1996; Chung et al., 2008). Once activated, the receptor undergoes two forms of desensitization: 1) 'acute desensitization', a reduction in current amplitude during a single exposure of a stimulus; or 2) 'tachyphylaxis', a pharmacological and functional diminuation of peak current amplitudes in response to repeated exposure to a stimulus (Koplas et al., 1997). The ability of the receptor to undergo an overall diminished response as a result of prolonged or repeated exposure to a stimulus ignited the investigation of this receptor as a possible therapeutic target for various pain conditions (i.e. inflammation, hyperalgesia, neuropathic pain and etc.). However, understanding molecular mechanisms influencing the activity of the TRPV1 receptor and its involvement in various pathological pain states has also prompted considerable research.

Capsaicin, an agonist of TRPV1, is found in hot peppers, and has been commonly used to activate the receptor under experimental conditions. The agonist is a lipophillic compound that diffuses through the plasma membrane and binds to intracellular sites on the N- and C- termini (Jung et al., 1999 & 2002); in particular, the TM2-TM3 linker, and on the channel-lipid interface of TM2 andTM3 (Jordt & Julius, 2002), thereby allowing entry of current. The increase in pain sensitivity in various pathological pain conditions has been attributed to amplification of TRPV1 activity (reviewed by Patapoutian, Tate & Woolf, 2009). Sensitization of the TRPV1 receptor has been shown to be caused by proinflammatory mediators that activate PKC and results in the enhancement in the membrane current of TRPV1 receptors (Dai et al., 2004; Moriyama et al., 2005; Sikand & Prekumar, 2007; Zhang et al., 2007). In addition, increased trafficking of the receptor to the membrane through retrograde transport of neurotrophic factors (i.e. nerve growth factor and glial cell line-derived neurotrophic factor) and the activation of phosphoinositide 3-kinase signalling cascade has also been shown to enhance TRPV1 activity in DRG neurons (Amaya et al., 2004; Zhuang et al., 2004; Stein et al., 2006; Malin et al, 2006; Zhu & Oxford, 2007).

Interestingly, sensitization of TRPV1 receptors has been reported in the STZ-induced diabetic model in rodents (Hong & Wiley, 2005; Pabbidi et al., 2008 a & b). These studies

confirmed that diabetic rodents exhibit a reduced latency to withdrawal from a noxious thermal stimulus compared to age-match controls. This sensitization was correlated with increased inward currents and increased duration of capsaicin evoked currents in DRG neurons. However, these observations were based on animal models with long standing diabetes induced by STZ. Whether STZ can influence ion channel regulation and pain sensitivity remains poorly understood. It has been observed that DRG neurons in culture exposed to STZ exhibited a higher level of ROS and an increase in TRPV1 activity and expression, suggesting STZ as a possible confound variable to consider (Pabbidi et al., 2008a).

Oxidative stress caused by pharmacological exposure to reducing and oxidizing agents has been demonstrated to covalently modify cysteine residues in the extracellular and cytoplasmic domains of TRPV1 and in turn, influenced the sensitivity of the receptors in HEK293 cells (Susankova et al., 2006; Chuang & Lin, 2009). These studies revealed that oxidative modification, in particular, via the formation of inter-cysteine disulfide bonds within the cytoplasmic domain of the ion channel, sensitized the receptor in response to repeated applications of capsaicin. However, it remains unclear from these studies if hyperglycemia-dependent oxidative stress can induce changes in TRPV1 activity directly or as a consequence of the length of the disease.

1.5 Objectives

This thesis investigated the role of high glucose (HG)-induced oxidative stress on P2X and TRPV1 receptors expressed on mice lumbar sensory neurons in culture. The central hypothesis of this thesis is that elevated cytoplasmic ROS, or oxidative stress, as a result of high glucose for seven days will potentiate P2X and TRPV1 membrane currents. To examine this, I used electrophysiological, live imaging and biochemical tools to understand the function of these neurotransmitter receptors in sensory neurons under control and high glucose conditions. The ultimate objective of this study is to generate data that may help in the development of new therapeutic targets for the treatment of diabetic-related complications.

CHAPTER 2

ELECTROPHYSIOLOGICAL CHARACTERIZATION OF TRPV1-MEDIATED CURRENTS IN SENSORY NEURONS: MONOPHASIC AND BIPHASIC RESPONSES

2.1 Introduction

Capsaicin (CAP) is a lipophilic chemical present in hot chili peppers that activates a nonselective cation channel, the transient receptor potential vanilloid 1 channel (TRPV1, Tominaga & Tominaga, 2003). CAP binds to various sites within the TRPV1 receptor, including the TM2-TM3 intracellular linker, within the channel-lipid interface in the TM2 and TM3 membrane domains, and at intracellular sites in the N- and C-termini (Jung et al., 2002; Jordt & Julius, 2002; Immke & Gavva, 2006). Exposure of sensory neurons of the dorsal root ganglion to CAP increases intracellular calcium levels through the TRPV1 receptor, which is highly permeable to the divalent cation (Wood et al., 1988). In addition, once TRPV1 channels have been activated by CAP, they can undergo desensitization that affects the magnitude and kinetics of the CAP-evoked currents. Different desensitization mechanisms cause the reduction of the CAP-evoked current. Acute desensitization is responsible for the current transient observed during a single application of CAP, while tachyphylactic desensitization is observed during repetitive CAP applications over a period of time (Petersen et al., 1996; Oh et al., 1996; Koplas et al., 1997). Desensitization by tachyphylaxis is dependent on extracellular Ca^{2+} and leads to the successive diminishment of TRPV1-mediated currents (Koplas et al., 1997). It has been proposed that a rise in intracellular Ca^{2+} activates calcineurin and leads to de-phosphorylation and desensitization of the CAP-sensitive TRPV1 channel (Docherty et al., 1996; Mohapatra & Nau, 2005; Por et al., 2010), providing a physiological role for desensitization of TRPV1 channels following repetitive activation in blocking pain transmission.

In the present study, I observed that lumbar DRG neurons could be subdivided in two groups based on the type of responses elicited following repetitive CAP applications. TRPV1 currents displayed two types of responses: (1) a 'monophasic response' with a diminuation of peak current typical of tachyphylactic desensitization; or (2), a 'biphasic response' composed of an initial gradual potentiation of the peak current followed by its desensitization by tachyphylaxis. Interestingly, replacing external calcium by barium, a divalent cation commonly used to examine Ca^{2+} regulation of ion channels, revealed three characteristics of the TRPV1 responses to repeated CAP applications: 1) the potentiation of CAP-evoked current in the biphasic response was independent of extracellular calcium, 2) the time course of the current potentiation was dependent on extracellular calcium, and 3) the tachyphylactic desensitization of CAP-evoked currents was dependent on extracellular calcium, as described by previous reports

(Koplas et al., 1997; Docherty et al., 1997; Mohapatra & Nau, 2005). In addition, with the help of an inhibitor of the beta isoform of protein kinase C (PCKβ), I demonstrated that activity of this kinase is essential for the potentiation part of the biphasic response following repetitive CAP application. I also confirmed that the two different patterns of CAP responses were present in calcitonin-gene related peptide (CGRP)-positive neurons, consistent with the role of these neurons in pain perception and processing also in the adult. Collectively, these results demonstrate that TRPV1 from nociceptive lumbar DRG neurons display two forms of responses following successive CAP applications, suggesting that they may play different roles in mechanisms of pain.

2.2 Materials and Methods

2.2.1 Primary culture

All experiments were approved by the University of Saskatchewan's Animal Research Ethics Board, and adhered to the Canadian Council on Animal Care guidelines for humane animal use. Primary DRG cultures for neonatal C57BL/6 mice between postnatal day 1 to 3 (P1-P3) were used for the preparation of the dissociated DRG cultures. The methods used to dissociate the neurons were similar to the protocol used to dissociate superior cervical ganglion neurons (Campanucci et al., 2008; McFarlane & Cooper, 1992). Briefly, mice were euthanized by cervical transection in a sterile environment. Lumbar (L1 to L5) DRGs from the spinal cord were collected into a Petri dish with serum-containing media (L15 supplemented with vitamins, cofactors, penicillin-streptomycin and 5 mM glucose, and 10% horse serum). Small iris scissors were used to trim the remaining nerve roots from the ganglia. Once cleaned, the ganglia were submerged in an enzymatic solution (0.1% trypsin; Worthington, Freehold, NJ, USA) buffered with 1 M of HEPES (pH 7.4), and incubated at 37°C for 30- 45 min in a water bath for enzymatic dissociation of ganglia.

Next, DRGs were mechanically dissociated by using a fire-polished pipette. The dissociated neurons were rinsed twice with serum-containing media to inactivate trypsin. The resulting cell suspension was transferred to growth media consisting of L-15 supplemented with vitamins, cofactors, penicillin-streptomycin, 5% rat serum, 7S Nerve Growth Factor (10 ng/ ml) and 5 mM of glucose. The neurons were plated on laminin-coated coverslips attached to

modified 35 mm tissue culture dishes. Cells were maintained at 37°C in a 95% air and 5% CO_2 environment and fed every 3-4 days with growth media. To eliminate non-neuronal cells, cultures were treated with cytosine arabinoside (10μM; Sigma, St. Louis, MO, USA) from day 1 to day 3. Cells were allowed to recover from stress and axotomy for one week prior to any treatment or experimental procedure. Cultured neurons were maintained in medium containing either 5 mM glucose (control) or switched to 25 mM glucose (high glucose), for 1 week. Two weeks following the day of culture, cells were subjected to electrophysiological experiments.

The method used to isolate of DRG neurons from adult transgenic CGRP- eGFP CD1 mice were similar to the protocol used in this study with minor modification based on the protocol used by Burkey et al. (2004). Briefly, dissected and cleaned lumbar DRGs were incubated at 37 °C in 0.1% collagenase (Worthington type 2, Calbiochem, San Diego, CA, USA) for 30 minutes and then 0.1% trypsin for an additional 30 minutes. After titration, the dissociated neurons were rinsed with Dulbecco's modified eagle medium (DMEM, Gibco, Carlsbad, CA, USA) containing 10% horse serum, followed by resuspension in growth media containing DMEM supplemented with vitamins, cofactors, penicillin-streptomycin, 5% rat serum, 7S Nerve Growth Factor (10 ng/ ml) and 5 mM of glucose. Maintenance of cultured adult neurons was as indicated for neonatal neurons.

2.2.2 Electrophysiology

CAP-evoked current were recorded using the whole-cell patch-clamp technique (Hamill et al., 1981). Patch pipettes were made from borosilicate glass (WPI, Sarasota, FL, USA) using a vertical puller (PC 10; Narishige Scientific Instrument Lab., Tokyo, Japan) and were fire-polished with the help of a microforge (MF 900; Narishige). Micropipettes had a resistance of 5-10 MΩ when filled with intracellular recording solution, and formed gigaseals of 1-8 GΩ. Recording electrodes were filled with the following solution (in mM): 60 KAc, 70 KF, 5 NaCl, 1 $MgCl_2$, 1 $CaCl_2$, 2 MgATP, 10 EGTA, and 10 HEPES, and pH was adjusted to 7.2 with KOH. Junction potentials were cancelled at the beginning of the experiment. The external solution contained (in mM): 140 NaCl, 5.4 KCl, 0.33 NaH_2PO_4, 0.44 KH_2PO_4, 1 $MgCl_2$, 1 $CaCl_2$, 10 HEPES, and 5 glucose, and pH was adjusted to 7.4 with NaOH. For barium experiments, 1-2 mM of $BaCl_2$ was used in place of $CaCl_2$ in the external solution. Whole-cell currents or

membrane potentials were recorded at room temperature with the aid of an Axopatch 200B amplifier (Axon Instruments Inc., Union City, CA, USA) equipped with a 1 GΩ headstage feedback resistor and sampled at 5 kHz with a Digidata 1440A. Voltage clamp protocols, data acquisition and analysis were performed using pCLAMP 10 software (Axon Instruments) and Origin 7 (OriginLab Coorporation, Northampton, MA, USA). Once the whole-cell configuration was achieved, cells were allowed to stabilize for 5 min before recording. Using a fast-step perfusion system, neurons were exposed to control or CAP (5 μM) solution at a rate of 1 mL/min. Tetrotodoxin (TTX, 1 μM) was added to both control and CAP solutions to continuously block voltage-gated sodium channels.

2.2.3 Statistics

For comparisons of mean current density, I used the unpaired Student's t-test. Comparisons of ratio of ionic currents, integrated current area, and percentages, I used the non-parametric Mann-Whitney test.

2.3 Results

2.3.1 Repetitive applications of capsaicin elicited monophasic and biphasic responses of TRPV1-mediated currents

Small to medium sized dissociated lumbar DRG neurons (15-25 pF) were exposed to a series of brief repetitive applications of CAP (5 μM for 1 s, at 15 s intervals) to examine TRPV1-mediated currents in whole-cell patch clamp mode. Consistent with previous reports, repetitive applications of CAP evoked a monophasic response depicted by the run-down (or desensitization) of the TRPV1-mediated peak currents in some cells (Fig. 2-1A, left column). Unexpectedly, given the same number of CAP applications, a subgroup of neurons displayed a biphasic response depicted by a gradual potentiation of the peak TRPV1-mediated current followed by its desensitization (Fig. 2-1A, right column). To quantify the number of cells exhibiting the monophasic or biphasic response, I used a threshold criterion of 50% increase in

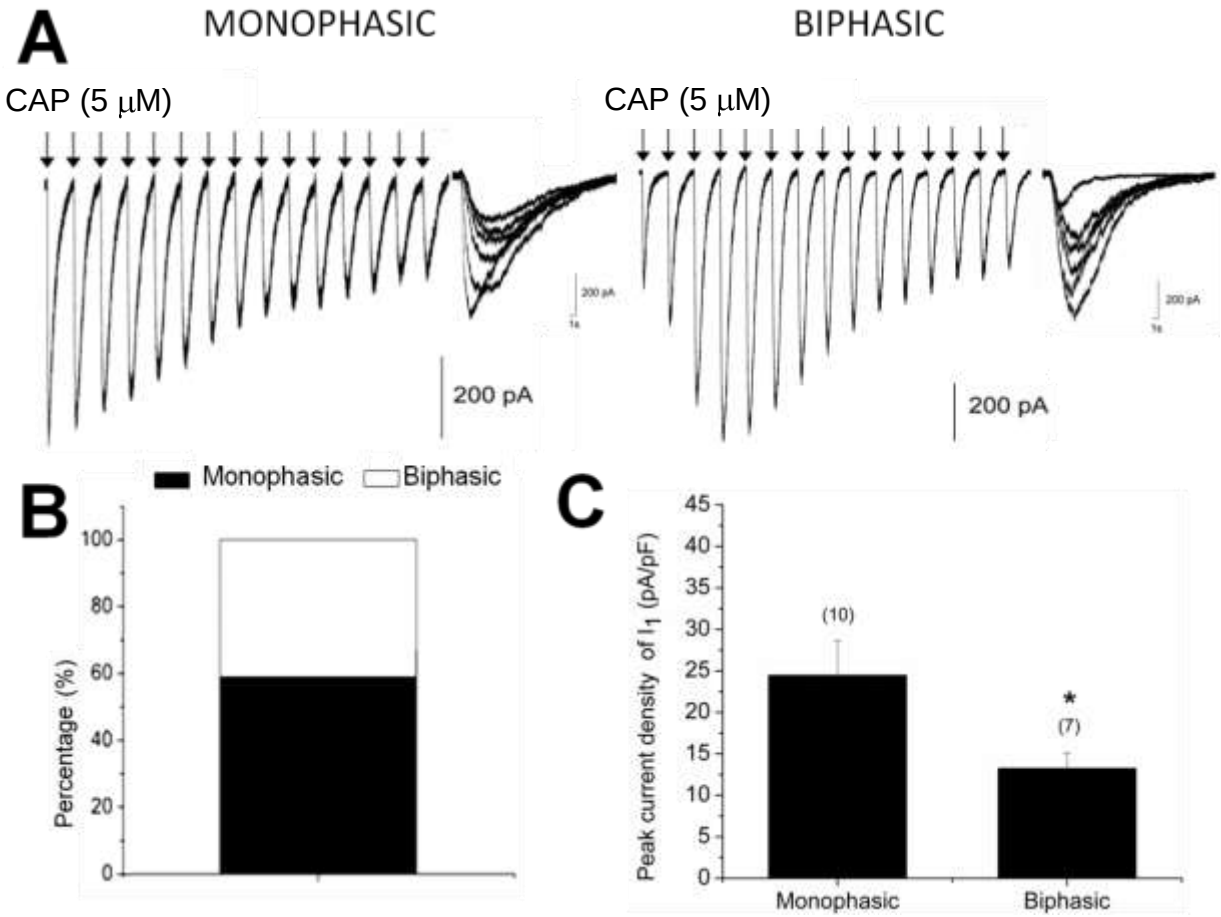

Figure 2-1. **CAP-evoked currents show two distinct types of responses**. **(A)**Whole-cell currents in voltage clamp mode were obtained from a series of 1 s capsaicin (CAP) applications (5 μM) at 15 s intervals. Repetitive CAP applications revealed two behaviors in TRPV1-mediated currents. The monophasic response displayed desensitization in TRPV1-mediated currents (left column, n=10), while the biphasic response displayed an initial potentiation followed by desensitization in TRPV1-mediated currents (left column, n=7). Superimposition of these current traces is represented following every 3 applications of CAP. **(B)** The distribution of these responses is summarized in the bar graph where a greater percentage of neurons displayed the monophasic response (black) relative to the biphasic response (white)**. (C)** The bar graph summarizes the mean±SEM initial peak current density (I_1) of the monophasic and biphasic response following the first CAP application. * indicates statistical significance between conditions at a level of $p<0.05$. The distinct difference in I_1 between responses helped to identify the two responses to repetitive CAP-applications during the course of the experiments.

the maximum peak current relative to the initial peak amplitude of CAP-evoked currents in a series. This 50% value was used because it best represented the two distinct types of responses in TRPV1 activity. Based on the threshold criterion, monophasic responses occurred in 59% of cells (n=10) while biphasic responses occurred in 41% of cells recorded (n=7, Fig. 2-1B). To determine whether there was a difference in the magnitude of the initial CAP-evoked current (I_1), I compared the peak current density of I_1 in monophasic and biphasic responses (Table 2-1). The peak current density of I_1 for the monophasic response was significantly larger, being approximately twice the amplitude of I_1 in the biphasic response ($p<0.05$, Fig. 2-1C). This distinctive characteristic helped to identify the two responses to repetitive CAP-applications during the course of the experiments.

2.3.2 Differential effects of calcium on the desensitization of the monophasic and the potentiation of the biphasic response

To further characterize these responses, I next investigated mechanisms that underlie the two types of responses. I first concentrated on the tachyphylactic desensitization observed over repetitive CAP applications. Because both responses displayed this run-down effect, I compared the degree of desensitization of the maximal CAP-evoked current (I_{max}) in both the monophasic and biphasic responses relative to the 15th CAP-evoked current (I_{15}) generated in the series (Table 2-1). Interestingly, the degree of desensitization, quantified as the percent change in the peak current density and integrated area, were not statistically different between responses ($p>0.05$), suggesting that it may involve the same mechanisms. Previous works have shown that the degree of tachyphylaxis was dependent on calcium entry (Koplas et al., 1997; Mohapatra et al., 2003). Therefore, to confirm that the tachyphylactic desensitization of CAP-evoked currents observed in the present study was consistent with previous reports in DRG neurons, Ca^{2+} ions were replaced in the extracellular solution by equal concentration of barium (Ba- ECF, Fig. 2-2). Interestingly, all neurons recorded in Ba-ECF revealed whole-cell currents that were smaller in peak amplitude (Fig. 2-2A, right) compared to neurons recorded in standard calcium extracellular solution (Ca- ECF; Fig. 2-2A, left). The CAP-evoked responses displayed a gradual potentiation that did not desensitize but remained stable in peak current amplitudes, suggesting that calcium entry is required to initiate desensitization of the receptor, as previously described.

Table 2-1 **Quantitative assessment and comparison of current traces between monophasic and biphasic responses from DRG neurons.**

		Monophasic (n=10)	**Biphasic (n=7)**
Peak current density (pA/pF)	$\mathbf{I_1}$	24.47±4.46	13.25±1.87*****
	$\mathbf{I_{max}}$	22.68±5.41	28.44±4.56
Integrated area (pC)	$\int I_1$	$0.40x10^7 \pm 0.11x10^7$	$0.22x10^7 \pm 0.40x10^7$
	$\int I_{max}$	$0.53x10^7 \pm 0.11x10^7$	$0.66x10^7 \pm 0.18x10^7$
Percentage of desensitization (%)	$\mathbf{I_{max}/I_{15}}$	55.88±5.25	61.02±4.00

* Indicates statistical difference between type of responses at a level of $p<0.05$.

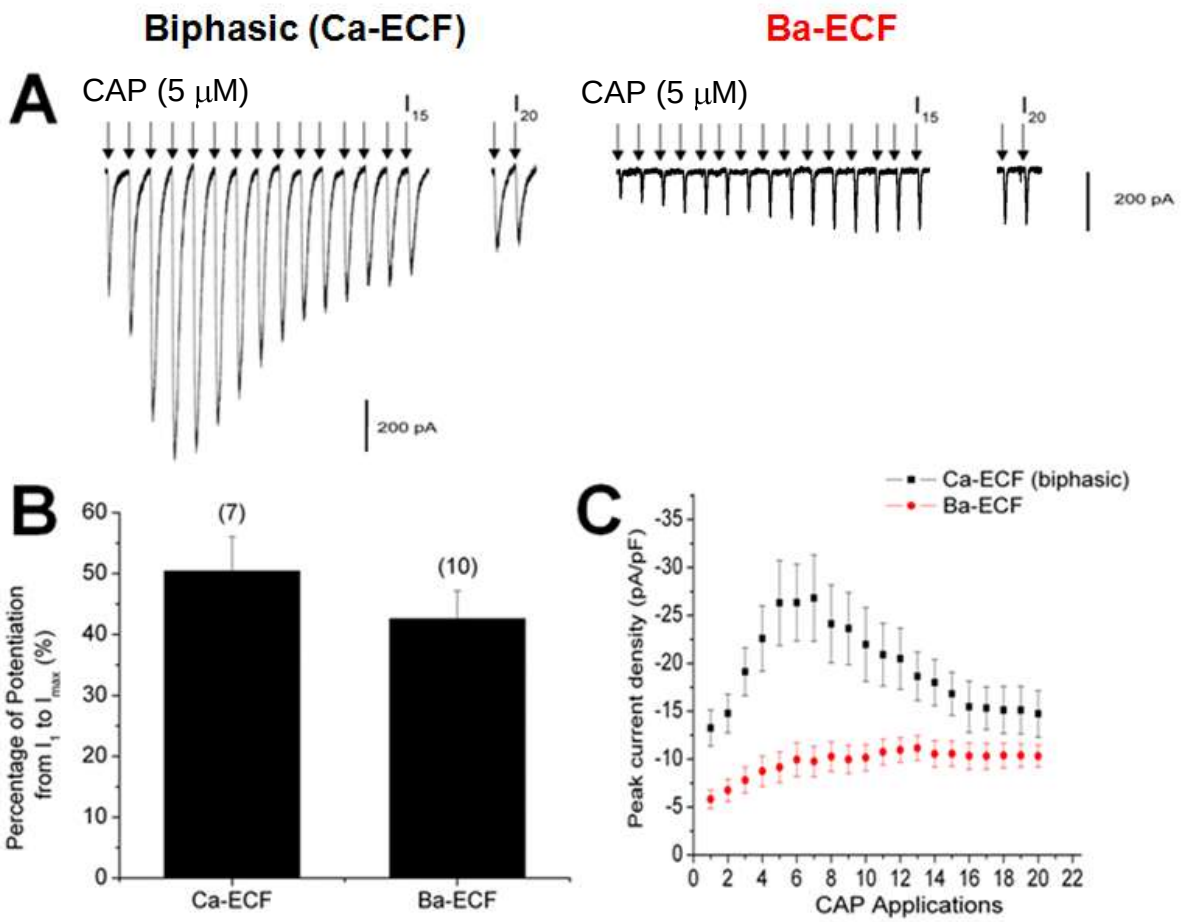

Figure 2-2. **Potentiation in CAP-evoked currents is independent of extracellular calcium**. **(A)** Whole-cell currents for cells recorded in Ba-ECF (1-2 mM) were obtained from a series of 1s CAP applications (5 μM) at an interval of 15 s in voltage clamp mode. In all neurons, successive CAP applications revealed a gradual potentiation in CAP-evoked current that leveled off and remained stable in current traces (left column). For comparison of potentiation, the right column is an example of whole-cell current traces of a neuron displaying the biphasic response recorded in Ca-ECF. **(B)** Bar graph summarizes the mean±SEM percentage of potentiation (measured as of the percentage of I_1 relative to I_{max}). **(C)** The scatter plot summaries the mean ±SEM peak current density for neurons recorded in Ba-ECF (red) and those that displayed the biphasic response in Ca-ECF (black), revealing a greater number of CAP applications to reach I_{max} peak amplitude for neurons recorded in Ba-ECF relative to those in Ca-ECF.

To study the dependency of potentiation of CAP-evoked current on Ca^{2+} ions, I recorded in extracellular Ba-ECF. Under this recording condition, all neurons displayed current traces that potentiated, similar to the potentiation in the biphasic response recorded in standard Ca-ECF (Fig. 2-2A). To quantify the degree of potentiation in TRPV1-mediated currents for cells recorded in Ba-ECF and Ca-ECF, I examined the percentage increase from I_1 relative to I_{max}. Despite the reduction of charge generated in CAP-evoked currents recorded in Ba-ECF, the percentage of potentiation was no different from neurons recorded in Ba-ECF or Ca-ECF, (Fig. 2-2B). In addition, it appeared that the onset for maximal peak current amplitude required more CAP applications for neurons recorded in Ba-ECF (i.e. 13 applications), relative to those recorded in Ca-ECF (i.e. 6 applications, Fig. 2-2C). Arguably, this would suggest that the time course to generate maximal current amplitude is influenced by Ca^{2+} or Ca^{2+} dependent signalling. These findings revealed that although the removal of extracellular calcium strongly diminished the magnitude of CAP-evoked current, which was therefore mediated by barium and monovalent cations it did not affect the degree of potentiation in CAP-evoked currents. Thus, these data suggest that other molecular mechanisms may be involved in the potentiated phenomenon.

2.3.3 Inhibition of PKCβ activity revealed two types of responses following repetitive activation of TRPV1 receptors

The resulting potentiation of CAP-evoked currents in all neurons recorded under Ba-ECF, which was similar to the biphasic response though smaller in amplitude, suggested that other factors may be involved in the generation of the potentiation response. The gradual potentiation of CAP-evoked currents observed in the present study is similar to that previously reported from studies where PKC activity was pharmacologically manipulated. An increase of PKC activity sensitized TRPV1 channels, causing a gradual increase in the channel's membrane current following repetitive CAP-induced activation (Prekumar & Ahern, 2000; Vellani et al., 2001; Bhave et al., 2003). However, this effect was never reported under control conditions. It is possible that PKC may be involved in the biphasic response observed in the present study. To examine the influence of PKC activity on the biphasic response in neurons, I first examined the

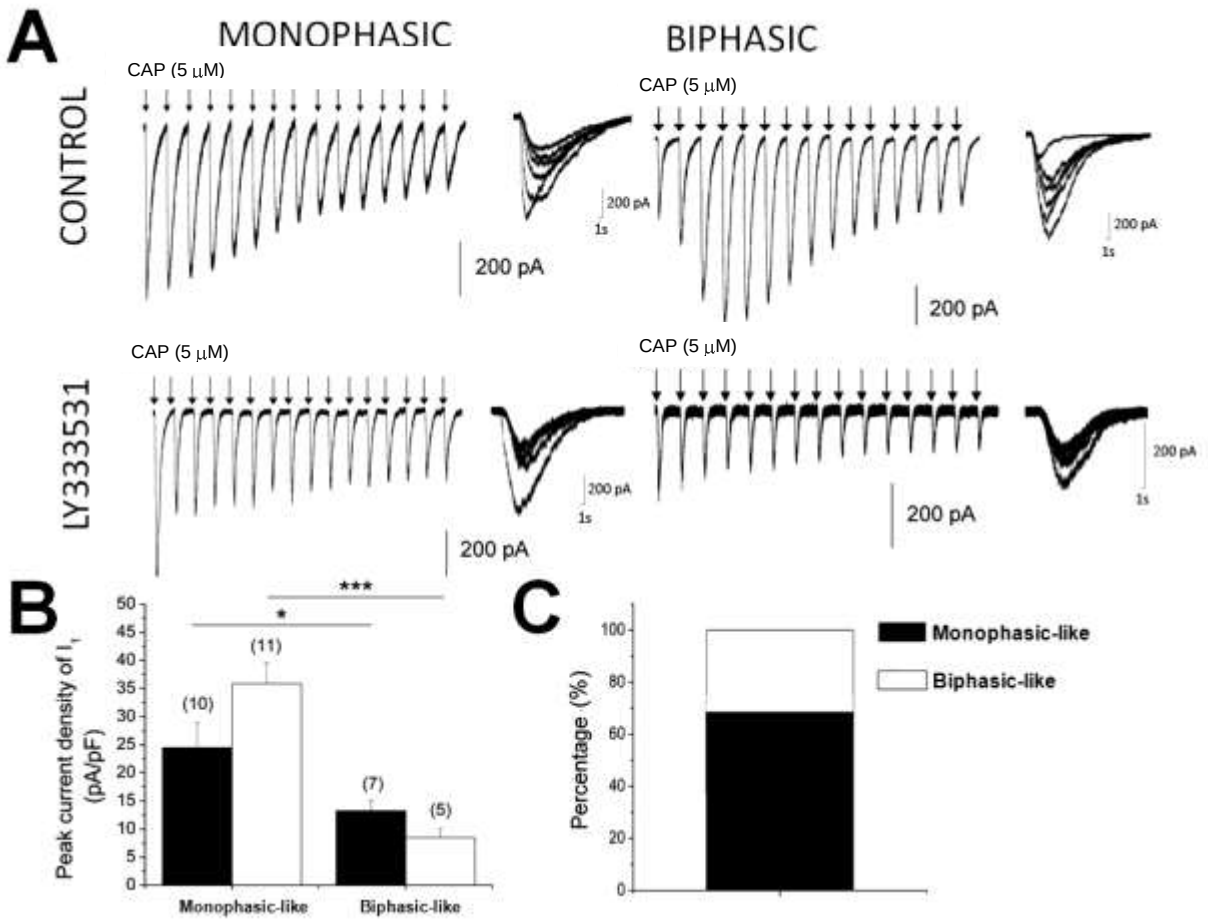

Figure 2-3. **Inhibition of PKCβ activity appeared to prevent the potentiated phenomenon in the biphasic response and greatly enhanced desensitization in the monophasic response. (A)** Whole-cell current traces were obtained from DRG neurons exposed to an intracellular solution containing the PKCβ inhibitor, LY333531 (50 nM), and exposed to repeated applications of CAP (1 s, 15 s). Top row, represents whole-cell current traces from cells recorded in control conditions displaying the monophasic (left) or biphasic (right) response. Bottom row, represents whole-cell current traces from cells exposed to LY333531 displaying characteristics of the monophasic response (left) or the biphasic response (right). **(B)** Bar graph summaries mean±SEM peak current density for the initial CAP-evoked current in neurons displaying the monophasic and biphasic response in control and LY333531 conditions. * indicates statistical significance between conditions at a level of $p<0.05$. *** indicates statistical significance between conditions at a level of $p<0.001$. **(C)** The bar graph is a summary of the distribution of the monophasic-like response (black) and the biphasic-like response (white) from the sampled population of neurons treated with LY333531**.**

influence of the PKC beta isoform (PKCβ), a classic PKC isoform that hasbeen shown to play a role in a number of diabetic complications (Geraldes & King, 2010), particularly in diabetic hyperalgesia (Kim et al., 2003; Tahara et al., 2006).

DRG neurons were exposed intracellularly to the PKCβ inhibitor, LY333531 (50 nM), through the patch-pipette Once whole-cell configuration was achieved, the inhibitor was allowed to diffuse into the cell for 5 minutes prior to recording currents. Following a series of brief CAP applications (1 s, 15 s), whole-cell currents exhibited two types of responses that displayed distinctive initial peak current amplitudes (I_1) that allowed identification of the monophasic and biphasic responses (Fig. 2-3A and B). The first type was consistent with the monophasic response ("monophasic-like"), where the first application of CAP caused a large inward current that underwent tachyphylactic desensitization (Fig. 2-3A, bottom row, left), although, the desensitization rate seemed to be faster and more robust than that observed in the monophasic response under control conditions (Fig. 2-3A, top row). The second response ("biphasic-like") displayed a small inward current that desensitized following successive applications of CAP. Interestingly, when the distribution of the responses in neurons treated with LY333531 were examined, 68% of neurons displayed the monophasic-like response, while 32% displayed the biphasic-like response, similar to the distribution of monophasic and biphasic neurons in the control condition (Fig. 2-3C).

To further evaluate the influence of LY333531 on TRPV1 currents, the degree of desensitization and potentiation were examined (Table 2-2). Interestingly, the degree of desensitization, measured as the percentage of I_{15} relative to I_{max}, for the monophasic-like response was nearly two-folds greater with LY333531, displaying a 76.0±3.41% decrease in maximal current response, while only a 44.1±5.25% decrease in maximal current response was revealed for the control condition (Fig. 2-4A). Conversely, the biphasic-like response for neurons treated with LY333531 displayed a similar degree of desensitization (32.0±10.8%) that was not significantly different from the biphasic response in control (39.0±4.0%, p=0.8765), suggesting that the desensitization for the biphasic-like response was unaffected by the PKCβ inhibitor. To evaluate the influence of LY333531 on the potentiation phenomenon, the degree of potentiation measured as the percent increase from I_1 to I_{max} was evaluated between biphasic neurons from the control and LY333531 treated conditions. It was revealed that LY333531 prevented the potentiation that occurred in control neurons that displayed the biphasic response,

Table 2-2 **Quantitative assessment and comparison of current traces between monophasic and biphasic responses from DRG neurons under control and LY333531 conditions**.

		Control		**LY333531**	
Parameters		**Monophasic (n=10)**	**Biphasic (n=7)**	**Monophasic (n=11)**	**Biphasic (n=5)**
Peak current density (pA/pF)	I_1	24.47±4.46	13.25±1.87*****	35.88±3.67	8.46±1.59 *******
	I_{max}	22.68±5.41	28.44±4.56	35.88±3.67	8.89±1.53***
Percentage of desensitization (%)	I_{max}/I_{15}	44.1±5.25	39.0±4.0	76.0±3.41$^{\$\$\$}$	32.0±10.8
Percentage of potentiation (%)	I_{max}/I_1		50.4±5.65		7.47±5.51$^{\$}$

***** indicates a significant difference between behaviours within treatment conditions at a level of $p<0.05$

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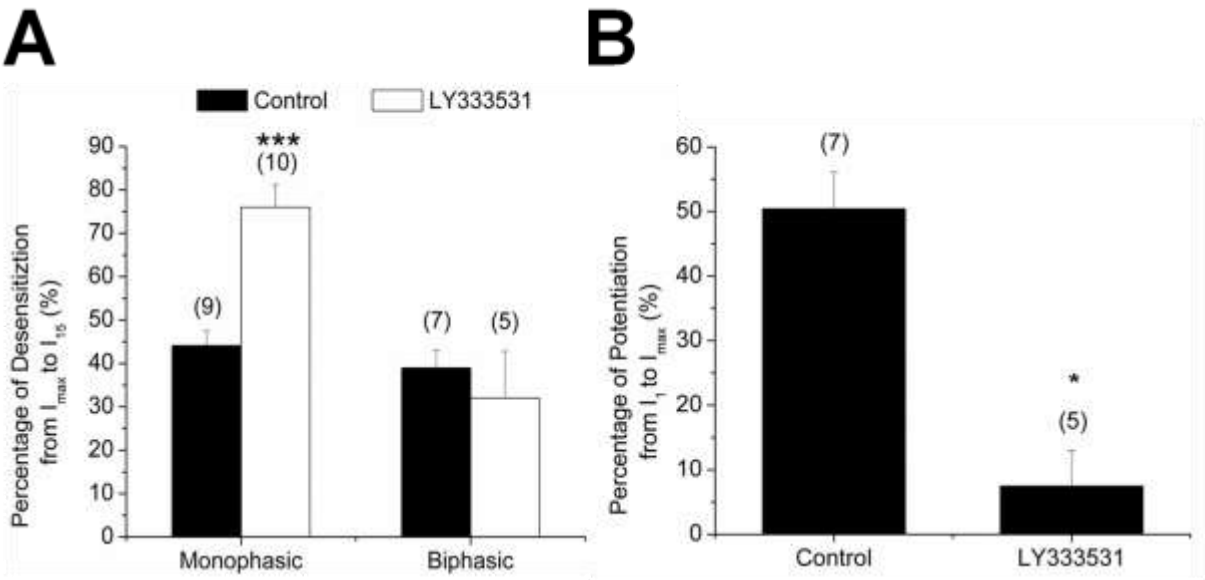

Figure 2-4. **Inhibition of PKCβ activity altered the sensitivity of TRPV1 receptors influencing the monophasic and biphasic response**. Bar graphs (**A** and **B**) summarizes the mean±SEM percentage of desensitization for neurons displaying the monophasic or biphasic response in control (black) and LY333531 (white) conditions and the percentage of potentiation for neurons responding in a biphasic-like manner when treated with LY33531 and in the control condition. * indicates a statistical significance between conditions at a level of $p<0.05$.

revealing a 7.47±5.51% potentiation in current generated relative to 50.4±5.65% potentiation observed in control neurons (Fig. 2-4B). Collectively, these findings suggest that inhibition of PKCβ activity greatly affected the response of TRPV1 receptors upon CAP activation, influencing both the desensitization and potentiation characteristic of the monophasic and biphasic responses.

2.3.4 Adult DRG neurons also display the monophasic and biphasic responses

During maturation, differentiation into peptidergic and nonpeptidergic neurons is highly dependent on growth factors. In addition, it has been recently shown that TRPV1 expression is down-regulated in nonpeptidergic DRG neurons, while peptidergic DRG neurons maintain the receptor expression, suggesting that this subpopulation of neurons has a greater role in pain processing (Cavanaugh et al., 2011). To determine the relevance of my findings in neonates to adult mice, I investigated whether TRPV1 receptors expressed in DRG neurons from one-month old adult mice also exhibited the monophasic and biphasic responses. To do this, I used enhanced GFP under the control of the CGRP promoter in transgenic CD1 (tgCGRP-eGFP) mice, to generate DRG cultures allowing selective identification of CGRP+ peptidergic neurons. Similar to neonatal DRG neurons, whole-cell current traces from adult DRG neurons displayed either a monophasic or biphasic response (Fig. 2-5A). With the 50% threshold criterion, there was a greater distribution of cells displaying the monophasic response (nearly 75%) compared to those displaying the biphasic response (~25%, Fig. 2-5B). Collectively, these findings support that the types of responses observed are an inherent property of TRPV1 channels that exists postnatal and in mature mouse DRG neurons.

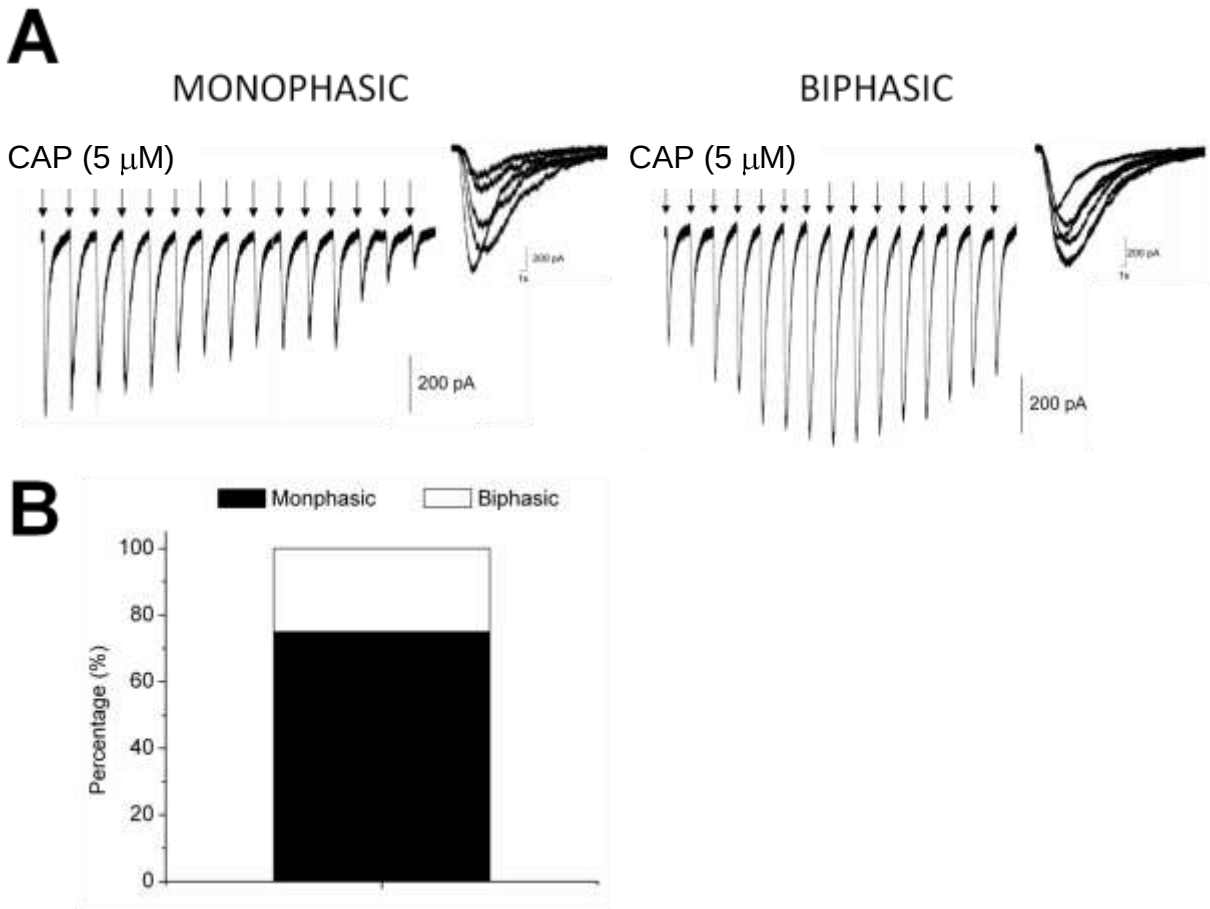

Figure 2-5. **Adult DRG neurons also displayed monophasic and biphasic responses in TRPV1 activity. (A)** In control *in vitro* conditions**,** whole-cell current traces from adult DRG neurons also exhibited monophasic and biphasic responses from repeated application of capsaicin. **(B)** Bar graph summarizes the distribution of monophasic (black) and biphasic (white).

2.4 Discussion

In this study, I characterized the responses of lumbar DRG neurons to repetitive CAP applications, which are mediated by the activation of TRPV1 receptors. The successive application of CAP evoked currents that underwent a run-down or tachyphylactic desensitization due to repeated exposure to the agonist. Tachyphylaxis is known to be mediated by calcium entry and the protein phosphatase calcineurin (Petersen et al., 1996; Docherty et al., 1997; Koplas et al., 1997). It was therefore unexpected to find that successive applications of CAP elicited two distinct responses in cultured DRG neurons: a 'monophasic response' characterized by the tachyphylactic desensitization of the original peak current and a 'biphasic response' characterized by the gradual potentiation of the CAP-evoked currents followed by tachyphylactic desensitization.

2.4.1 Effect of calcium on the monophasic and biphasic responses

The tachyphylaxis observed in both responses suggested that calcium entry was critical in initiating the desensitization of the channel upon repeated exposure to CAP (Docherty et al., 1996; Koplas et al., 1997). In fact, when calcium was substituted by barium in the extracellular solution, none of the neurons exhibited desensitization; rather, CAP-evoked currents were smaller in amplitude and displayed a gradual potentiation following successive applications of CAP. The findings from the barium experiment revealed three interesting characteristics of the TRPV1-mediated currents in DRG neurons: 1) the current potentiation of TRPV1 was independent of extracellular calcium, because it still occurred in Ba-ECF solutions; 2) the temporal progression of current potentiation was dependent on calcium or calcium-related signalling, since a greater number of CAP applications were required to reach maximal current amplitude in Ba-ECF; and interestingly, 3) initiation of tachyphylaxis was calcium-dependent, since it did not occur in Ba-ECF as was shown previously (Koplas et al., 1997; Docherty et al., 1997; Mohapatra & Nau, 2005).

From this study, I cannot rule out a possible role of intracellular calcium in TRPV1 activity in response to repetitive CAP applications. For instance, it was previously suggested

(Koplas et al., 1997) that the rise in intracellular calcium to a threshold level may facilitate calcium entry-induced desensitization of TRPV1 receptors. In future experiments, we plan to monitor and manipulate the level of intracellular calcium during repetitive CAP applications to test whether intracellular calcium affects the responses reported here.

Similarly, it is possible that the phosphatase calcineurin, which is activated by calcium and dephosphorylates TRPV1 receptors, leads to tachyphylactic desensitization (Mohapatra & Nau, 2005; Por et al., 2010). The inability to induce desensitization in TRPV1-mediated currents in the barium experiment could be attributed to the lack of calcium entry and the inability to activate calcineurin. However, additional experiments will need to delineate the role of intracellular calcium and calcineurin in regulating the monophasic and biphasic responses of the TRPV1 receptor.

2.4.2 Possible role of calcium-dependent signalling in the responses of DRG neurons to repetitive CAP applications

One should also consider the possibility that other mechanisms such as phosphorylation of the TRPV1 receptor by calcium-dependent PKC (Prekumar & Ahern, 2000; Velanni et al., 2001; Bhave et al., 2003), or CaMKII and ERK signalling (Zhang et al., 2010), could be involved in the gradual potentiation observed in the present study that were independent of pronociceptive effects influenced by inflammation or neurotrophic factors. Certainly, these endogenous activators can influence and regulate TRPV1 activity under physiological conditions. Under experimental conditions, activation of PKC in sensory neurons has been shown to increase the open probability of the TRPV1 receptor (Vellani et al., 2001). The fact that a lack of calcium in the Ba-ECF experiments led to potentiation in all the studied neurons, suggests that a calcium-regulated signalling protein may be responsible for the occurrence of the monophasic and biphasic responses. Therefore, I asked whether PKC may be responsible for the responses observed following repetitive CAP applications. I used the PKCβ inhibitor LY333531, because this PKC isoform is regulated by calcium and it was previously linked to attenuating diabetic hyperalgesia in STZ-induced diabetic rats (Kim et al., 2003). Neurons treated with LY333531 also displayed two types of responses that resembled the monophasic and biphasic responses, and were classified as monophasic-like and biphasic-like, respectively. The

monophasic-like response displayed a large inward current that desensitized by tachyphylaxis; however, the run-down was more robust than in the control condition. The mechanism for this robust effect may be attributed to the ongoing competition between kinases and phosphatases in regulating the TRPV1 channels. Reducing kinase activity may increase the probability of dephosphorylation of the TRPV1 receptor, and subsequently reduced sensitivity when exposed to the agonist. The biphasic-like response, however, displayed smaller currents that desensitized following a series of CAP applications. This suggests that phosphorylation of TRPV1 by PKCβ mediates the potentiation phenomenon in the biphasic response upon repeated applications of CAP. In addition, desensitization of the biphasic-like response could be related to similar mechanisms mediating the robust desensitization in the monophasic-like response. Conversely, it has been reported that PKC can endogenously activate TRPV1-mediated current in the absence of agonist application and similarly produce sensitization of membrane current upon repeated exposure to the PKC activator (Prekumar & Ahern, 2000). The findings of the present study would agree that the responses observed in TRPV1 activity was dependent on CAP-induced activation of the receptor as previously described (Vellani et al., 2001; Bhave et al., 2003) but the difference in monophasic and biphasic response may be influenced by the phosphorylated or dephosphorylated state of TRPV1.

While CAMKII and ERK signalling have demonstrated a time-dependent potentiation in TRPV1-mediated currents of at least 40 minutes between CAP applications (Zhang et al., 2010), and may be involved in the re-sensitization of the receptor (Jung et al., 2004), it unlikely that the potentiation observed in the present study was due to CAMKII-induced recovery of TRPV1 desensitization. The CAP protocol that I used to evoke TRPV1-mediated currents was shorter than is required to activate that pathway. Nevertheless, the present study demonstrated that under physiological conditions, repeated activation of TRPV1 by CAP can elicit two responses: a monophasic or biphasic response, which may be dependent on the phosphorylated or dephosphorylated state of the receptor prior to CAP application.

2.5 Conclusion

In summary, I have identified and characterized two types of responses in TRPV1-mediated currents: a monophasic and biphasic response, following a series of CAP applications in control *in vitro* conditions. To my knowledge, the biphasic response has not been identified in the literature of TRPV1 under control condition. In the absence of external calcium, all cells exhibited a gradual potentiation in TRPV1-mediated currents, similar to the potentiation observed in the biphasic response for cells recorded in the presence of external calcium. It should be considered that endogenous proteins (i.e. PKC, calcineurin, CAMKII) or intracellular calcium in DRG neurons can affect the sensitivity of TRPV1 receptors under control conditions and certainly reflected the two responses observed in this study.

CHAPTER 3

EFFECTS OF HIGH GLUCOSE ON P2X AND TRPV1 RECEPTORS IN MOUSE SENSORY NEURONS

3.1 Introduction

Diabetic sensory neuropathy (DSN) is associated with functional and morphological changes to the sensory nerves leading to reduction of nerve conduction velocity and the distal dying-back of axons (Polydefkis et al., 2004; Malik et al., 2005). Many patients with DSN display sensory abnormalities, including chronic pain or numbness in the distal limbs. In recent years, the idea has emerged that the dysregulation of glucose levels activate multiple distinct metabolic pathways leading to a singular end result: oxidative stress (reviewed by Brownlee, 2001; Vincent, et al., 2011). The increase in metabolic flux has been associated with dynamic changes in the mitochondrial morphology contributing to the overproduction of reactive oxygen species (ROS) in sensory neurons of the dorsal root ganglion (DRG, Russell et al., 2002; Vincent et al., 2002 & 2005a; Yu, Robotham & Yoon, 2006). However, it remains poorly understood what cellular and molecular mechanisms are influenced by ROS that contribute to the structural and functional changes in sensory neurons during diabetes.

Sensory DRG neurons are comprised of a heterogeneous population of neurotransmitter receptors. The purinergic (P2X) receptors and transient receptor potential 1 (TRPV1) channels have been identified as main players in sensory function and, in particular, of nociception (Chen et al., 1995; Caterina et al., 1997). P2X receptors are membrane ion channels activated by the binding of extracellular adenosine triphosphate (ATP, Grubb & Evans, 1999). Seven different subclasses have been reported (P2X1-7; North, 2002). In DRG neurons, a high proportion of mRNA transcript for the homodimeric P2X3 receptor are expressed, followed by P2X2 and heterodimeric P2X2/3 receptors (Chen et al., 1995; Lewis et al., 1995; Stebbing et al., 1998). The TRPV1 receptor is a polymodal detector activated by a number of stimuli: capsaicin, protons (pH), heat (>42°C), and arachidonic acid (Caterina et al., 1997). Although these receptors have been extensively studied with regards to pain processing, their role during hyperglycemic conditions remain poorly understood.

The present study investigated the role of high glucose-induced oxidative stress on P2X and TRPV1 receptors expressed on lumbar sensory neurons from neonatal mice in culture. In these experiments, an electrophysiological approach was used to examine the function of P2X and TRPV1 receptors exposed to repetitive agonist applications. In a series of ATP applications, no changes in the behavior of P2X-mediated currents were observed in neurons treated with high glucose relative to control. As denoted in Chapter 2, repeated applications of CAP evoked two

forms of responses in TRPV1-mediated currents: monophasic and biphasic responses (Chapter 2). In the present study, it was observed that these responses persisted following high glucose treatment. However, high glucose treatment caused an enhancement of TRPV1-medated currents in both type of responses, which was largely dependent on calcium entry. Because experiments with a ROS-sensitive dye revealed that high glucose-induced oxidative stress in DRG neurons, I next used an antioxidant mixture, which successfully reversed the high glucose-induced enhancement of currents. Lastly, I compared this effect of high glucose on TRPV1-mediated currents in DRG neurons from neonates and adult mice and found that although similar behaviours of TRPV1 currents were observed, the effects of high glucose displayed a differential degree of potentiation in adult DRG neurons.

3.2 Methods

3.2.1 Cell cultures

All experiments were approved by the University of Saskatchewan's Animal Research Ethics Board, and adhered to the Canadian Council on Animal Care guidelines for humane animal use. Primary DRG cultures for neonatal C57BL/6 mice between postnatal day 1 to 3 (P1-P3) were used for the preparation of the dissociated DRG cultures. The methods used to dissociate the neurons were similar to the protocol used to dissociate superior cervical ganglion neurons (McFarlane & Cooper, 1992; Campanucci et al., 2008). Briefly, mice were euthanized by cervical transection in a sterile environment. Lumbar (L1 to L5) DRGs from the spinal cord were collected into a Petri dish with serum-containing media (L15 supplemented with vitamins, cofactors, penicillin-streptomycin and 5 mM glucose, and 10% horse serum). Small iris scissors were used to trim the remaining nerve roots from the ganglia. Once cleaned, the ganglia were submerged in an enzymatic solution (0.1% trypsin; Worthington, Freehold, NJ, USA) buffered with 1 M of HEPES (pH 7.4), and incubated at 37°C for 30- 45 min in the water bath for enzymatic dissociation of ganglia.

Next, DRGs were mechanically dissociated by using a fire-polished pipette. The dissociated neurons were rinsed twice with serum-containing media to inactivate trypsin. The resulting cell suspension was transferred to growth media consisting of L-15 supplemented with vitamins, cofactors, penicillin-streptomycin, 5% rat serum, 7S Nerve Growth Factor (10 ng/ ml)

and 5 mM of glucose. The neurons were plated on laminin-coated coverslips attached to modified 35 mm tissue culture dishes. Cells were maintained at 37°C in a 95% air and 5% CO_2 environment and fed every 3-4 days with growth media. To eliminate non-neuronal cells, cultures were treated with cytosine arabinoside (10μM; Sigma, St. Louis, MO, USA) from day 1 to day 3. Cells were allowed to recover from stress and axotomy for one week prior to any treatment or experimental procedure. Cultured neurons were maintained in medium containing either 5 mM glucose (control) or switched to 25 mM glucose (high glucose), for 1 week. Two weeks following the day of culture, cells were subjected to electrophysiological experiments.

The method used to isolate of DRG neurons from adult transgenic CGRP- eGFP CD1 mice were similar to the protocol used in this study with minor modification based on the protocol used by Burkey et al. (2004). Briefly, dissected and cleaned lumbar DRGs were incubated at 37 °C in 0.1% collagenase (Worthington type 2, Calbiochem, San Diego, CA, USA) for 30 minutes and then 0.1% trypsin for an additional 30 minutes. After titration, the dissociated neurons were rinsed with Dulbecco's modified eagle medium (DMEM, Gibco, Carlsbad, CA, USA) containing 10% horse serum, followed by resuspension in growth media containing DMEM supplemented with vitamins, cofactors, penicillin-streptomycin, 5% rat serum, 7S Nerve Growth Factor (10 ng/ ml) and 5 mM of glucose. Maintenance of cultured adult neurons was as indicated for neonatal neurons.

3.2.2 ROS Levels and Oxidative stress

To measure cytoplasmic ROS levels, I used the redox- sensitive dye CM-H_2DCFDA (Molecular Probes, Burlington, Ontario, Canada), an acetoxymethyl (AM) ester. Cultures were incubated for one hour at 37°C with medium containing CM-H_2DCFDA (10μM) and subsequently washed five times with standard extracellular solution (see below). The cultures were then placed on the stage of an inverted epifluorescent microscope (Axiovert 200) and viewed through 40X oil-immersion objective (Zeiss) at room temperature. To obtain fluorescent images, I excited the cultures with 450 nm wavelength. To quantify the fluorescent intensity, the difference in intensity of the region of interest (neuronal cell body, excluding the nucleus) to the background was taken.

3.2.3 Electrophysiology

CAP-evoked current were recorded using the whole-cell patch-clamp technique (Hamill et al., 1981). Patch pipettes were made from borosilicate glass (WPI, Sarasota, FL, USA) using a vertical puller (PC 10; Narishige Scientific Instrument Lab., Tokyo, Japan) and were fire-polished using a microforge (MF 900; Narishige). Micropipettes had a resistance of 5-10 MΩ when filled with intracellular recording solution, and formed gigaseals of 1-8 GΩ. Recording electrodes were filled with the following solution (in mM): 60 KAc, 70 KF, 5 NaCl, 1 $MgCl_2$, 1 $CaCl_2$, 2 MgATP, 10 EGTA, and 10 HEPES, and pH was adjusted to 7.2 with KOH. For experiments using antioxidants, 500 μM of the antioxidant α-lipoic acid and 1000 U/mL of catalase was prepared fresh on the day of the experiment and dissolved in intracellular solution. Intracellular solution dialysis of 15 minutes was necessary to reduce cytoplasmic ROS levels as described previously by Campanucci et al. (2010), prior to the start of the experiment.

Junction potentials were cancelled at the beginning of the experiment. The external solution contained (in mM): 140 NaCl, 5.4 KCl, 0.33 NaH_2PO_4, 0.44 KH_2PO_4, 1 $MgCl_2$, 1 $CaCl_2$, 10 HEPES, and 5 glucose, and pH was adjusted to 7.4 with NaOH. For barium experiments, 1-2 mM of $BaCl_2$ was used in place of $CaCl_2$ in the external solution. Whole-cell currents or membrane potentials were recorded at room temperature with the aid of an Axopatch 200B amplifier (Axon Instruments Inc., Union City, CA, USA) equipped with a 1 GΩ headstage feedback resistor. and sampled at 1kHz and 5 kHz for ATP and CAP experiments, respectively, with a Digidata 1440A. Voltage clamp protocols, data acquisition and analysis were performed using pCLAMP 10 software (Axon Instruments) and Origin 7 (OriginLab Coorporation, Northampton, MA, USA). Once the whole-cell configuration was achieved, cells were allowed to stabilize for 5 min before recording. Using a fast-step perfusion system, neurons were exposed to control or agonist solutions [CAP (5 μM) or ATP (100 μM)] at a rate of 1 mL/min. Tetrotodoxin (TTX, 1 μM) was added to both control and CAP solutions to block voltage-gated sodium channels.

3.2.4 Statistics

To compare mean fluorescent intensities, I used the unpaired student's t- test. For current ratios, integrated area and percentages, the Mann Whitney nonparametric test was used. To

compare proportion of neurons in each condition, I used the Fisher's exact test (P1-P2). For comparison among multiple treatments, one-way ANOVA supplemented with Tukey's post hoc analysis was used.

3.3 Results

3.3.1 Increased oxidative stress in DRG neurons exposed to high glucose

Previous studies reported that exposure of DRG neurons to high glucose causes the accumulation of cytoplasmic ROS and the induction of oxidative stress (Russell et al., 2002; Vincent et al., 2002 & 2005a). To test whether high glucose in our hands induced oxidative stress, I exposed cultured DRG neurons to high glucose (25 mM) for 1 week and I monitored the cytoplasmic ROS levels with the ROS sensitive dye CM-H_2DCFDA. This experiment (Fig. 3-1) resulted in a significant two-fold increase in the mean pixel intensity in neurons exposed to high glucose (n=102) versus those in control conditions (5 mM of glucose; n=163).

3.3.2 Effects of high glucose on P2X-mediated currents in DRG neurons

Next, I examined the influence of high glucose (1 week) on the function of P2X receptors expressed in DRG neurons. To examine this, whole-cell currents were recorded following a series of ATP applications (100 µM, 1 s at 3 min intervals) on small to medium diameter (15-25 pF) DRG neurons. Applications of ATP predominantly evoked currents with fast-inactivating kinetics that resembled those carried by homodimeric P2X3 receptors, as described by Grubb & Evans (1999), in high glucose (Fig. 3-2A). To compare the influx of current over repetitive ATP applications in control and high glucose conditions, I examined the change in peak current (measured as a ratio of the 5^{th} ATP- evoked current to the first in the series), and the integrated area of the 5^{th} ATP-evoked current in the series. These comparisons did not show a significant change in ATP-evoked currents in control versus high glucose conditions (Fig. 3-2B and C). Collectively, these findings suggest that P2X-mediated currents in small to medium size lumbar DRG neurons were not affected by high glucose.

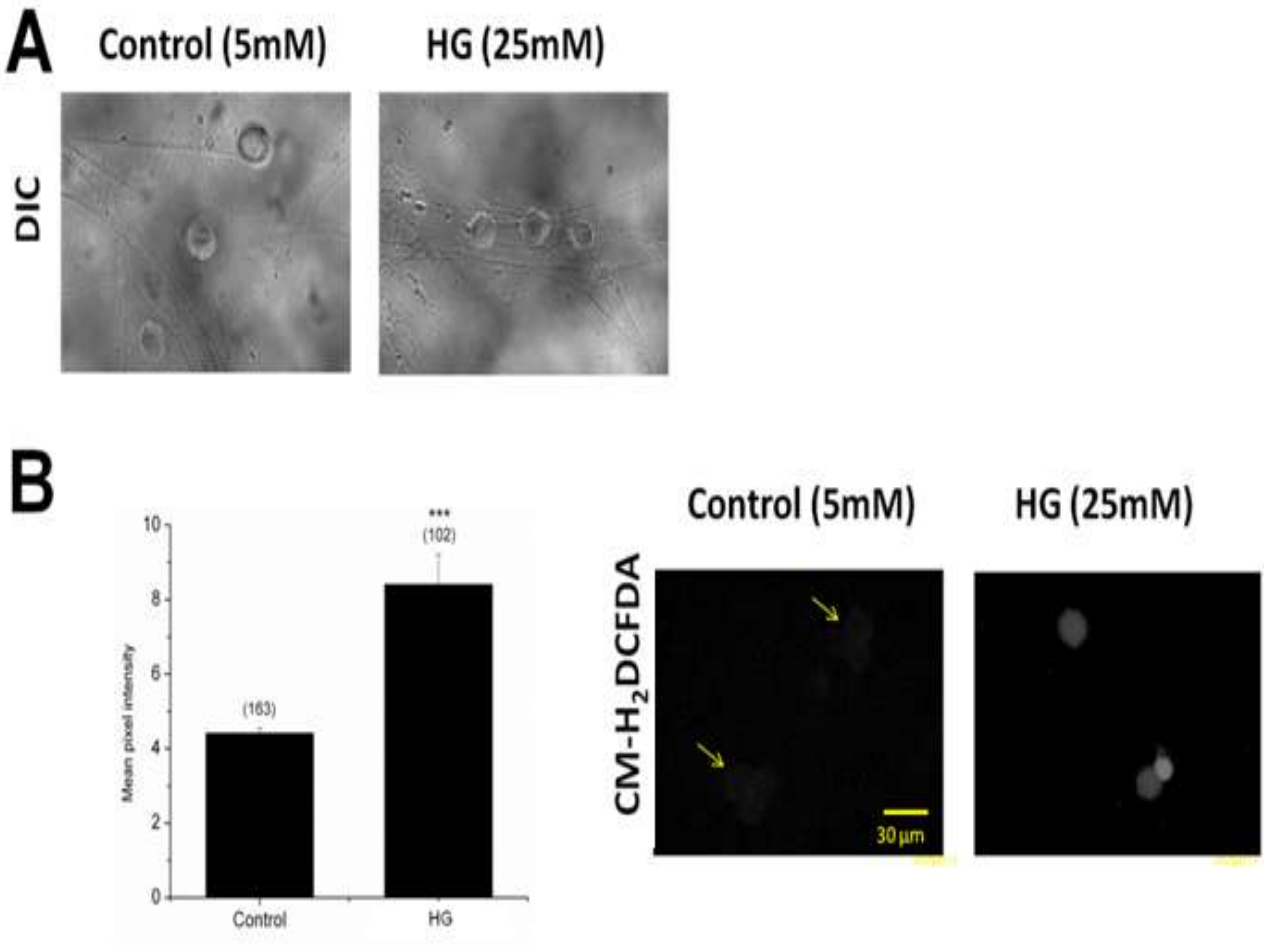

Figure 3-1. **DRG neurons exposed to high glucose for at least 7 days show elevated ROS levels. (A)** DIC images of cultured DRG neurons in control (5 mM, left column) and high glucose (HG, 25 mM) conditions. **(B)** The bar graph summarizes mean± SEM pixel intensity for ROS. *** indicates a statistical significance between conditions at $p<0.001$. Left, example of fluorescent images of DRG neurons from a different set of cultures that were treated with the ROS sensitive dye, CM-H_2DCFDA, in control (shown by the yellow arrows) and HG conditions to examine the differences in cytoplasmic ROS levels.

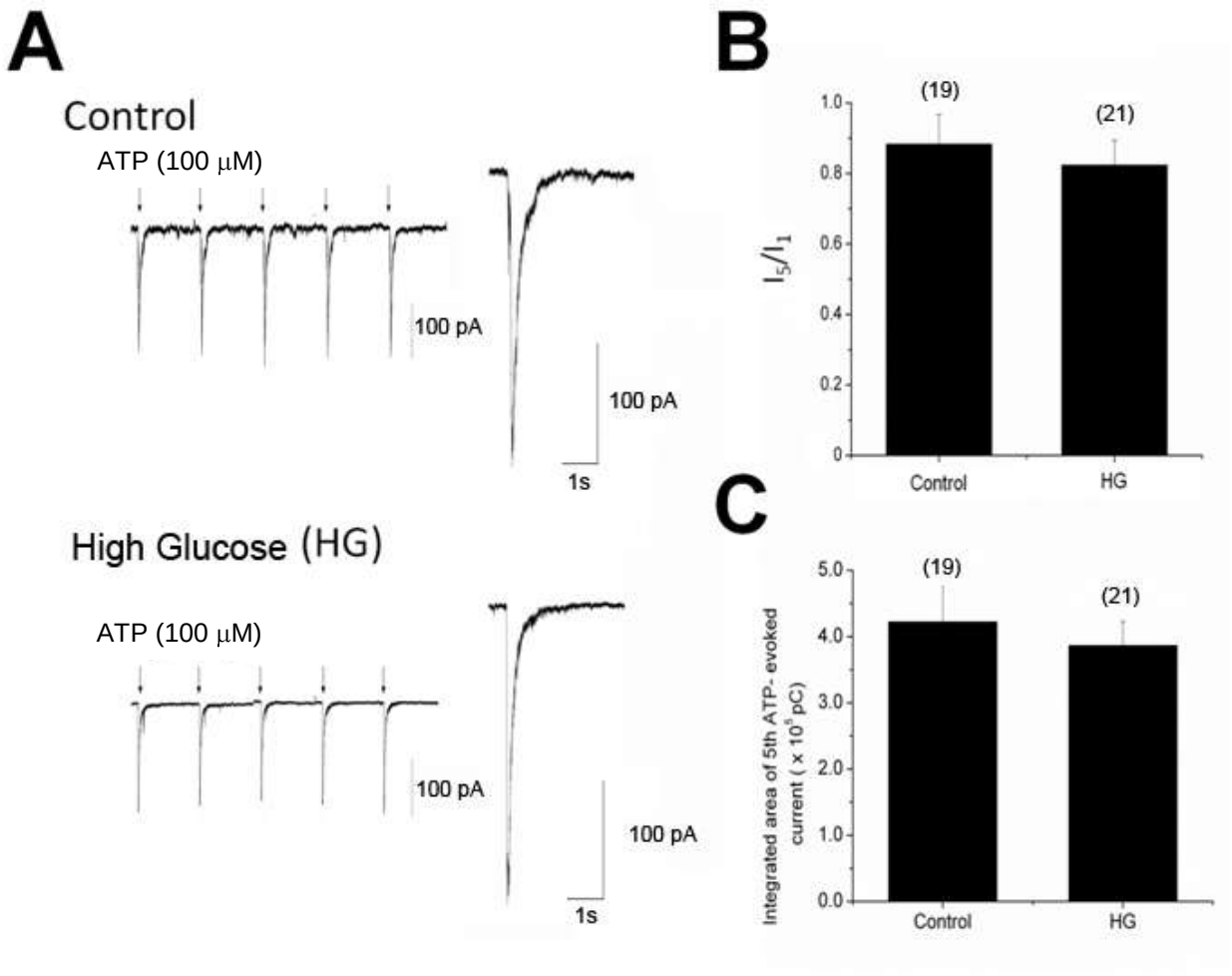

Figure 3-2. **One week of high glucose had no perceived effect on ATP-evoked currents in DRG neurons. (A)** A series of ATP application (100 μM, 1 s) every 3 minutes evoked a current response resembling P2X3 kinetics, which was fast-inactivating in control (top row) and high glucose (HG, bottom row) neurons in whole-cell voltage clamp mode. **(B)** and **(C),** The mean ± SEM current ratio of the 5th ATP-evoked current to the 1st ATP-evoked current and the mean± SEM integrated current area of the 5th ATP-evoked current revealed no significant differences between conditions using the Mann-Whitney nonparametric test.

3.3.3 Effects of high glucose on TRPV1-mediated currents in DRG neurons

I have previously reported that repetitive application of capsaicin (CAP, 5 μM, at 15 s intervals) elicited two types of responses in TRPV1-mediated currents: monophasic and biphasic responses (Chapter 2). Using the same protocol to apply CAP, I next examined the effects of high glucose on TRPV1-mediated currents using the whole-cell patch clamp technique. In these experiments, I observed the monophasic and biphasic responses in both experimental conditions (Fig. 3-3A). As described in Chapter 2, to distinguish cells exhibiting the biphasic response from monophasic response, I used a threshold criterion of a 50% increase in peak current amplitude from the maximum current amplitude (I_{max}) to the 1st (I_1) in the series. Based on this criterion, the distribution of the monophasic and biphasic responses in the high glucose condition was not significantly different from the control condition (Fig. 3-3B).

As described in Chapter 2, measurements of the peak current density of I_1indicated that the monophasic response was two-fold greater than I_1 for the biphasic response (Table 3-1). This effect persisted under high glucose conditions. However, it was observed that in high glucose the currents appeared enhanced (Fig. 3-3C). To quantify this potentiation, I examined the peak current density of I_{max} between conditions (note: in the monophasic response, I_{max} is I_1). Statistical analysis revealed a significant increase in peak current density of I_{max} in high glucose versus control for both responses (Fig. 3-4A). Interestingly, I noticed that under the high glucose condition, the duration of the current increased over a single CAP application. Therefore, to quantify this observed effect, I examined the integrated area of I_{max}. It was revealed that there was a significant increase in the integrated area of I_{max} in high glucose versus control conditions, in both the monophasic and biphasic responses ($p<0.05$, Fig. 3-4B). This suggested that in high glucose, the amount of current in both the monophasic and biphasic responses was enhanced. To examine if this enhancement in CAP-evoked currents had an affect on the parameters of the responses in high glucose, I compared the percentage of I_{max} to I_{15} to quantify the degree of tachyphylactic desensitization or I_1 to I_{max} in the biphasic response to quantify the degree of potentiation (Fig. 3-4C and D, respectively). Interestingly,

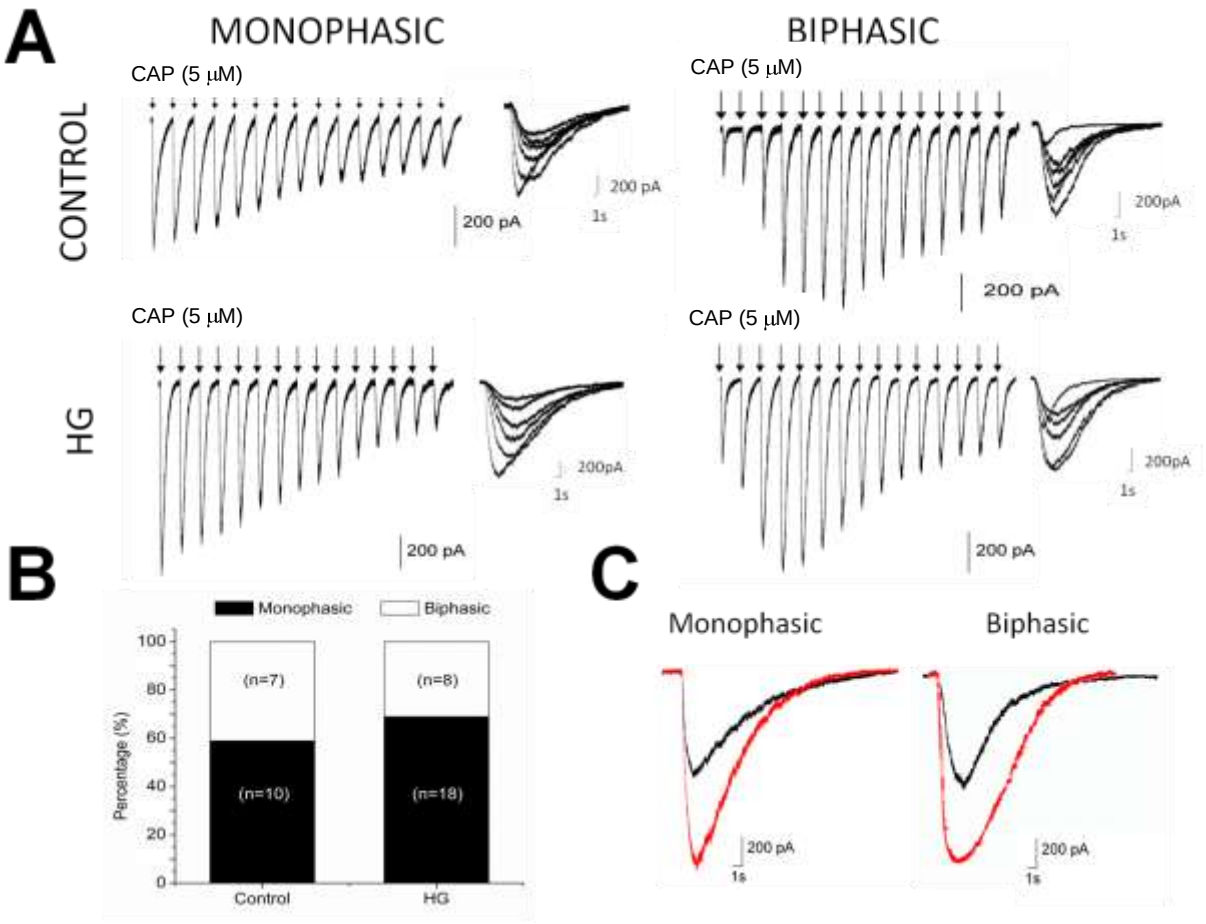

Figure 3-3. **CAP-evoked currents show two distinct types of responses in both control and high glucose conditions**. **(A)**Whole-cell currents in voltage clamp mode were obtained from a series of CAP applications (5μM, 1 s) at short intervals of 15 s revealing two behavioural responses in TRPV1 currents in control (top row) and high glucose (HG, bottom row) conditions. The monophasic response displayed desensitization in TRPV1- mediated currents (left column), while the biphasic response displayed a gradual potentiation followed by desensitization in TRPV1-mediated currents (right column) in both conditions. Superimposition of these current traces is represented following every 3 applications of CAP. **(B)** The bar graph summarizes the percentage of neurons exhibiting the monophasic (black) or biphasic (white) response in the sampled population from control and HG conditions. **(C)** The difference in the current generated between conditions is depicted by the overlap of I_{max} current traces from control (black) and HG (red) in the monophasic (left) and biphasic (right) responses.

Table 3-1. **Quantitative comparison between monophasic and biphasic in control and high glucose conditions.**

		Control		HG	
Parameters		**Monophasic (n=10)**	**Biphasic (n=7)**	**Monophasic (n=18)**	**Biphasic (n=8)**
Peak current density (pA/pF)	I_1	24.47±4.46	13.25±1.87*****	34.48±4.72	17.85±2.63 ******
	I_{max}	22.68±5.41	28.44±4.56	40.29±5.08$^{\$}$	47.24±6.38$^{\$}$
Integrated area (pC)	I_1	$0.40x10^7$ ±0.11 $x10^7$	$0.22x10^7$ ±0.40x 10^7	$0.91x10^7$ ±0.22 $x10^7$	$0.39x10^7$ ±0.07 $x10^7$
	I_{max}	$0.53x10^7$ ±0.11 $x10^7$	$0.66x10^7$ ±0.18x 10^7	$1.17x10^7$ ±0.22 $x10^7$ $^{\$}$	$1.52x10^7$ ±0.34 $x10^7$ $^{\$}$
Percentage of desensitization (%)	I_{max}/I_{15}	44.1±5.25	39.0±4.0	46.80±5.89	49.91±7.86
Percentage of potentiation (%)	I_{max}/I_1		50.4±5.65		60.63±4.27

***** indicates a significant difference between responses within treatment conditions at a level of $p<0.05$

$^{\$}$ indicates a significant difference between responses comparing treatment conditions at a level of $p<0.05$

****** indicates a significant difference between responses within treatment conditions at a level of $p<0.01$.

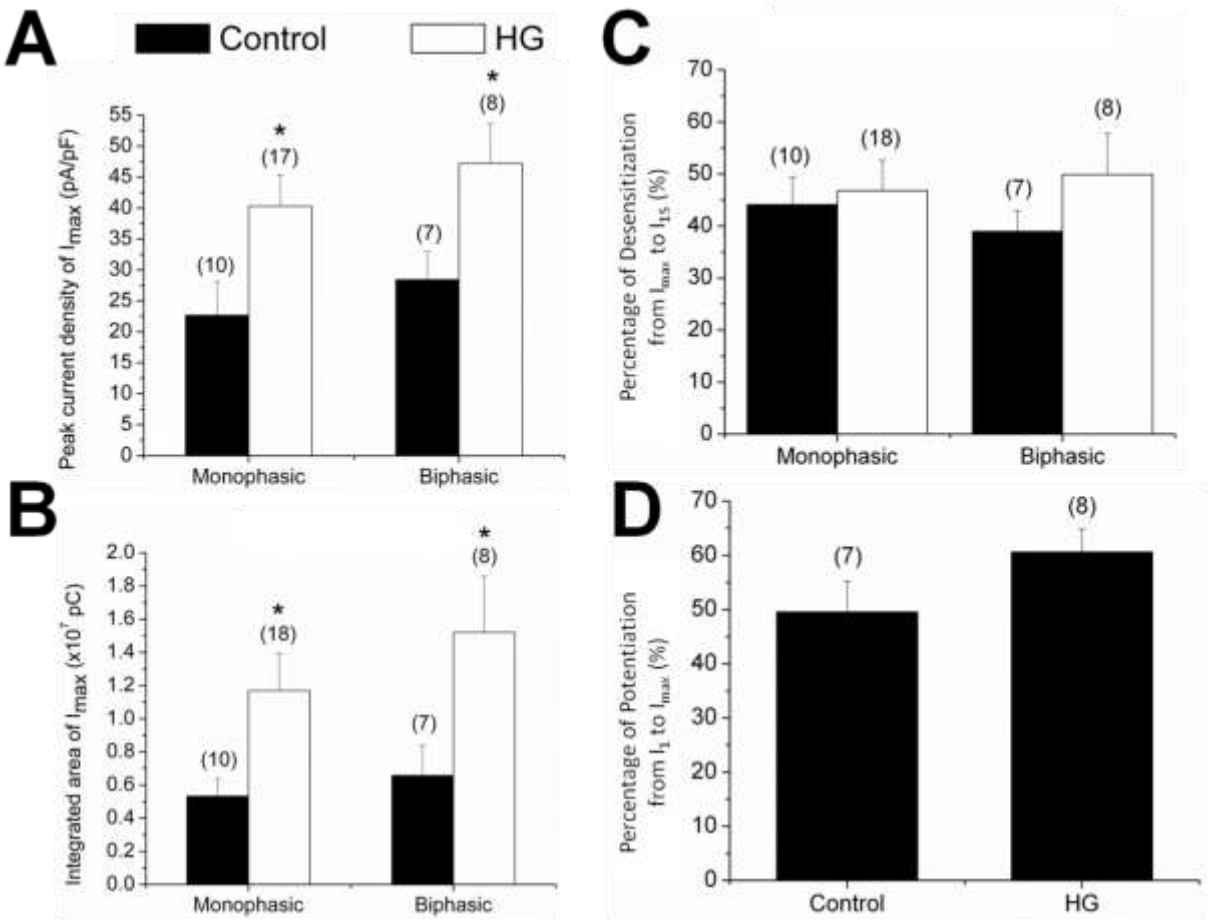

Figure 3-4. **High glucose enhanced TRPV1-mediated currents but had no perceived influence on the activity of the TRPV1 receptor.** The bar graphs summarizes the mean ± SEM peak current density **(A)** and integrated area **(B)** of I_{max} CAP-evoked current in neurons exhibiting the monophasic or biphasic response under control (black) and HG conditions (white). Neurons exposed to HG regardless of the type of response show a significant increase in peak current density and integrated current area at a level of $p<0.05$, *. Bar graphs (**C** and **D**) summaries the mean± SEM of the degree of desensitization (measured as the percentage of I_{max} relative to I_{15}) and the degree of potentiation (measured as the percentage of I_1 relative to $I_{max)}$ in control and HG conditions. Statistical analysis using Mann-Whitney nonparametric test revealed no significant differences between conditions.

this comparison showed no significant difference between conditions, suggesting that the mechanisms underlying the enhancement in the TRPV1-mediated currents under high glucose did not affect the mechanisms of desensitization and potentiation characteristic of the monophasic and biphasic responses.

3.3.4 The enhancement of the CAP-evoked current under high glucose is calcium-dependent

Next, I asked whether the enhancement in CAP-evoked currents was calcium dependent. Previous studies reported abnormal calcium homeostasis in sensory neurons from STZ-induced diabetic animals (Huang et al., 2002; Fernyhough & Calcutt, 2010). Whether Ca^{2+} carried by TRPV1 receptors contributed to this homeostatic imbalance during hyperglycemia remains to be determined. Therefore, I conducted an experiment in which Ca^{2+}in the extracellular fluid was substituted with the divalent cation barium (Ba^{2+}, Ba-ECF) to test the role of Ca^{2+} on CAP-evoked currents during high glucose. As previously shown in Chapter 2, all neurons displayed smaller currents that potentiated in Ba-ECF (Fig. 3-5A, right column). This phenomenon persisted in neurons in high glucose (n=14). Interestingly, the enhancement of CAP-evoked currents observed in high glucose when recording in Ca-ECF (Fig. 3-5A, left column) was absent in Ba-ECF. This was based on the comparison of the degree of potentiation in the biphasic response between control and HG conditions (Fig. 3-5B and C). This suggested that the enhancement of I_{max} in the biphasic response during high glucose was calcium dependent. The lack of desensitization in neurons recorded in Ba-ECF supports previous work demonstrating that the influx of calcium was necessary to desensitize TRPV1 receptors (Docherty et al, 1996; Koplas, 1997).

3.3.5 Antioxidant cocktail reversed the enhancement of CAP-evoked currents under high glucose

Since the central hypothesis of the present study that oxidative stress is the key mediator in the enhancement of CAP-evoked currents caused by high glucose, I investigated whether the enhancement of TRPV1-mediated current in high glucose could be prevented by reducing

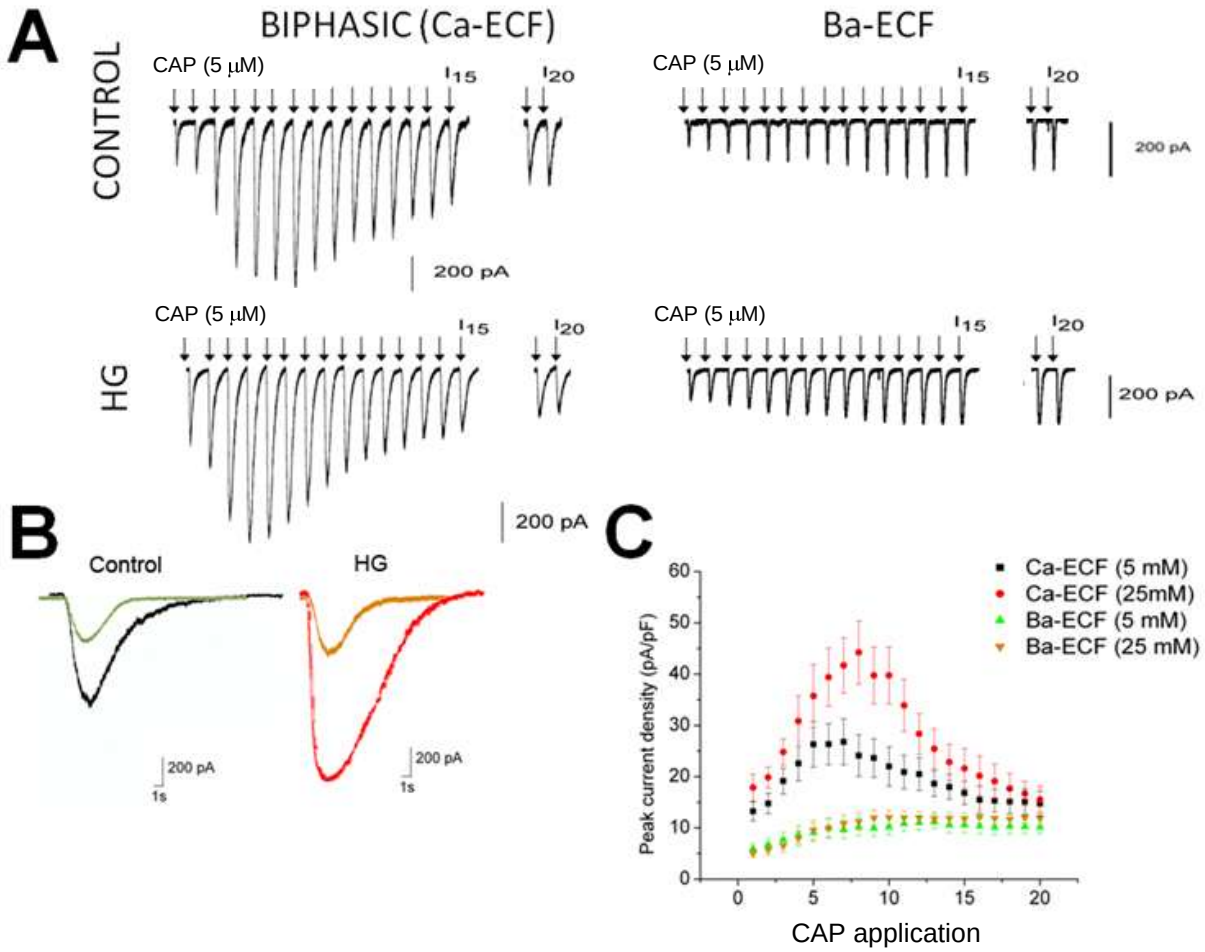

Figure 3-5. **Effects of high glucose on CAP-evoked current is calcium dependent. (A)** Barium, a divalent cation, was used to examine calcium dependency of TRPV1 currents under high glucose conditions. Whole- cell current traces from neurons in control (top row) and HG (bottom row) recorded in Ba-ECF revealed an initial potentiation (left column) that resembled the potentiation in the biphasic response for neurons recorded in Ca-ECF (right column). However, subsequent applications of CAP evoked current amplitudes that remained stable in amplitude shown at the 20th CAP-evoked current (I_{20}), which contrasted the desensitization observed in neurons recorded in Ca-ECF at I_{20}. **(B)** Superimposition of I_{max} CAP-evoked currents for control (left) and HG (right) neurons recorded in Ca-ECF (black and red current traces) compared to neurons recorded in Ba-ECF (green and orange current traces). **(C)** The scatter plot summaries the mean± SEM peak current density as a function of CAP applications for control (5mM) and HG (25mM) neurons recorded in Ca-ECF and Ba-ECF.

cytoplasmic ROS levels. An experiment was performed to determine whether an antioxidant cocktail (500μM of α-lipoic acid and 1000 U/mL of catalase, ALA), loaded into the recording electrode, would reverse the enhancement of TRPV1-mediated current observed in neurons under the high glucose condition. Interestingly, antioxidant-treated neurons recorded in whole-cell patch clamp mode exhibited an altered response that was not characteristic of the monophasic or biphasic response. CAP-evoked current recorded in the presence of antioxidant cocktail did not potentiate or desensitize. Rather, whole-cell current traces displayed an initial large inward current followed by stable CAP-evoked currents that were usually half the size of the initial current (Fig. 3-6A).

To evaluate this effect, I examined the peak current amplitude and integrated area of the 6^{th} CAP-evoked current in the series. The mean peak current density of the 6^{th} CAP-evoked current in control+ ALA was significantly decreased compared to control neurons. Remarkably, when neurons were exposed to HG+ ALA, the enhancement of I_6 was prevented and comparable to control conditions (Fig. 3-6B). Collectively, the data gathered from this experiment suggested that the enhancement of TRPV1-mediated currents under the high glucose condition can be reversed by reducing cytoplasmic ROS through intracellular application of antioxidants. However, since cytoplasmic redox state played a role in the type of responses (i.e. monophasic or biphasic) observed in DRG neurons, it suggests that these responses need to be further explored.

3.3.6 Adult DRG neurons also show enhancement of CAP- evoked currents under high glucose

During maturation, differentiation into peptidergic and nonpeptidergic neurons is highly dependent on growth factors. In addition, it has been recently shown that TRPV1 expression is down-regulated in nonpeptidergic DRG neurons, while peptidergic DRG neurons maintain receptor expression, suggesting that this subpopulation of neurons has a greater role in pain processing (Cavanaugh et al., 2011). To determine the relevance of my findings, I investigated whether TRPV1 receptors expressed in DRG neurons from one-month old adult mice also exhibited the enhancement of the CAP-evoked currents as a result of elevation in ROS by high glucose. To do this, I used enhanced GFP under the control of the CGRP promoter in transgenic CD1 (tgCGRP-eGFP) mice, to generate adult DRG cultures allowing selective identification of CGRP+ peptidergic neurons. Similar to neonates and as described in Chapter 2, whole-cell

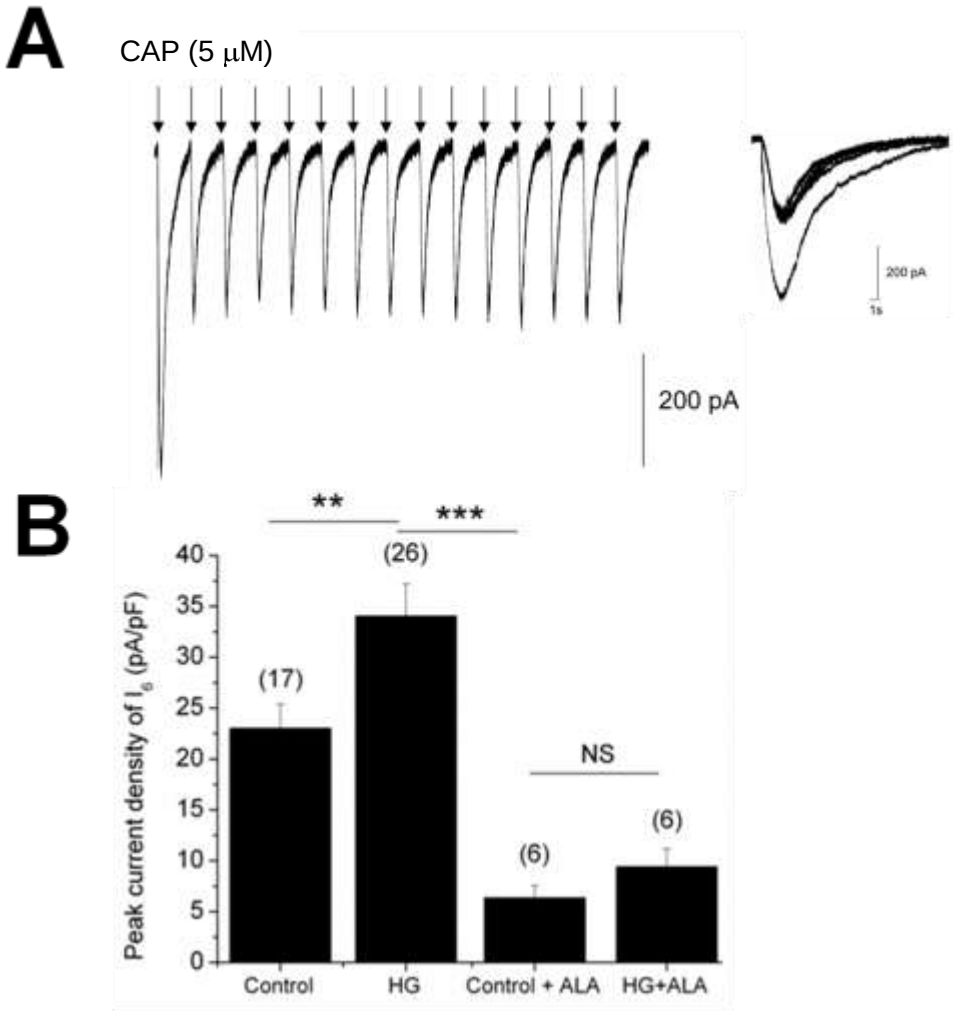

Figure 3-6. **High glucose-induced enhancement of CAP-evoked current is reversed with antioxidant treatment. (A)** Example of CAP-evoked current trace from neurons in the high glucose condition treated with an antioxidant mixture containing 500 μM of lipoic acid and catalase (HG+ALA), which displayed a response that was neither monophasic nor biphasic following a series of CAP applications. Superimposition of these current traces is represented following every 3 applications of CAP. **(B)** The mean± SEM peak current density of the 6th CAP-evoked current for neurons treated in control, HG, HG + ALA and control + ALA, respectively. ** p<0.01, *** p<0.001, NS indicates no significant difference between conditions.

current traces from adult DRG neurons in both control and high glucose conditions displayed either a monophasic or biphasic response (Fig. 3-7A).

Using the 50% threshold criterion, both control and high glucose conditions revealed a greater proportion of cells that displayed the monophasic response relative to the biphasic response (Fig. 3-7B); however, based on the small sample size, I cannot make any conclusive statements. To compare the enhancement in TRPV1-mediated currents during high glucose (Fig. 3-7C), I examined the peak current density and integrated area of I_{max} irrespective of the type of responses, due to the small sample size. The results in Fig. 3-8A, illustrate the mean peak current density of I_{max} CAP-evoked current in adult DRG neurons in control and high glucose conditions. Adult neurons in high glucose revealed a significant increase in the mean current density compared to that of control neurons ($p<0.05$, Fig. 3-8A), as shown in neonates (Fig. 3-4B). Conversely, no statistical differences were found for the integrated area of I_{max} CAP- evoked current in adult DRG neurons (Fig. 3-8B). The reduced potentiated effect observed in the integrated area of CAP-evoked current in adults compared to neonates may be attributed to developmental changes. However, similar to neonates, the degree of desensitization (Fig. 3-8C) and potentiation (Fig. 3-8D) was unaffected by the high glucose insult. This suggests that high glucose influences the magnitude of current in TRPV1 following repetitive activation, but does not influence mechanisms for desensitization or potentiation.

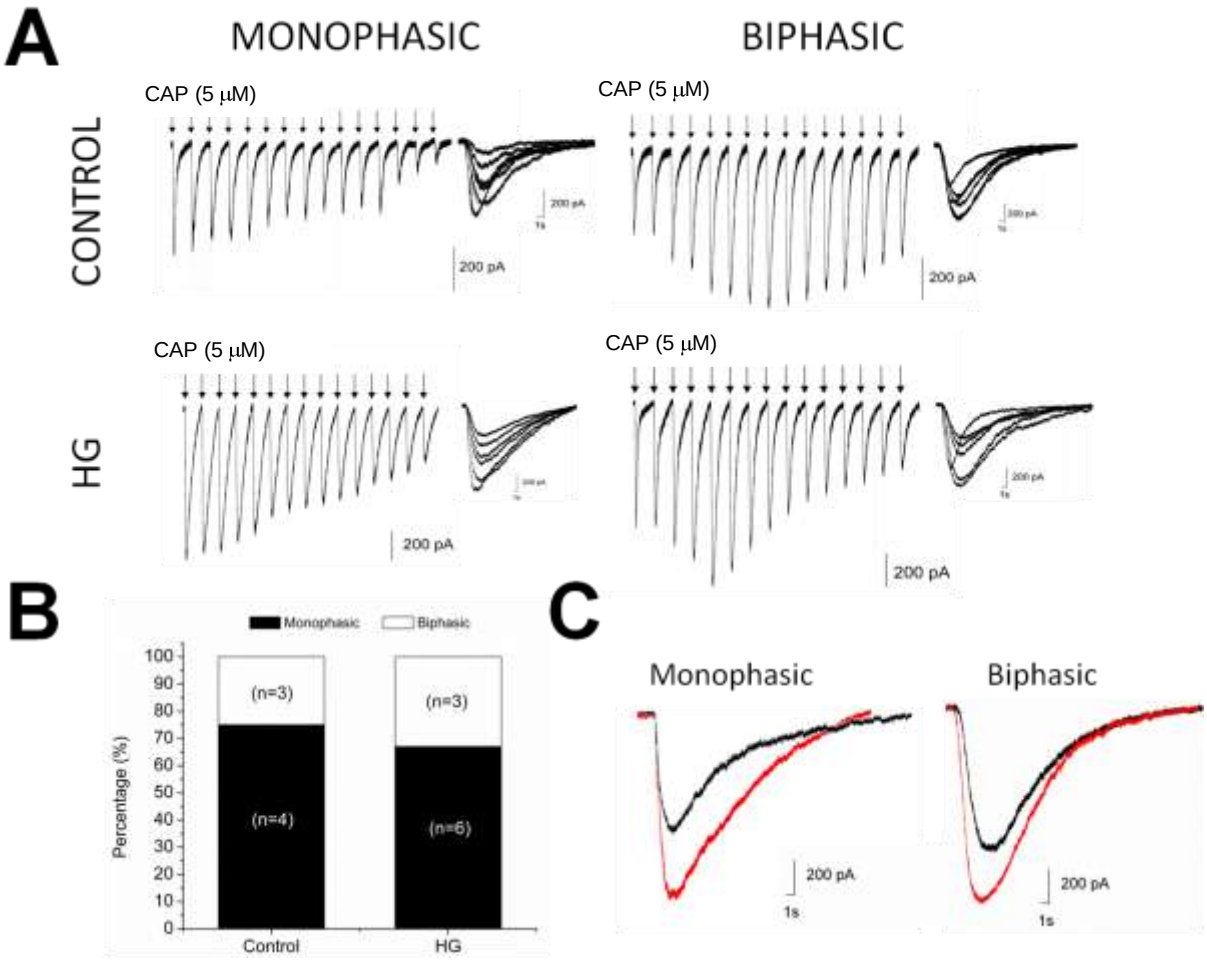

Figure 3-7. **Adult DRG neurons also displayed monophasic and biphasic responses in TRPV1-mediated currents under control and HG conditions. (A)** Whole-cell current traces from adult DRG neurons also exhibited monophasic and biphasic responses from repetitive applications of CAP. **(B)** Bar graph summarizes the distribution of monophasic (black) and biphasic (white) response in control and HG conditions. **(C)** The difference in current influx between conditions is depicted by the overlap of I_{max} current traces from control (black) and HG (red) in the monophasic (left) and biphasic (right) responses.

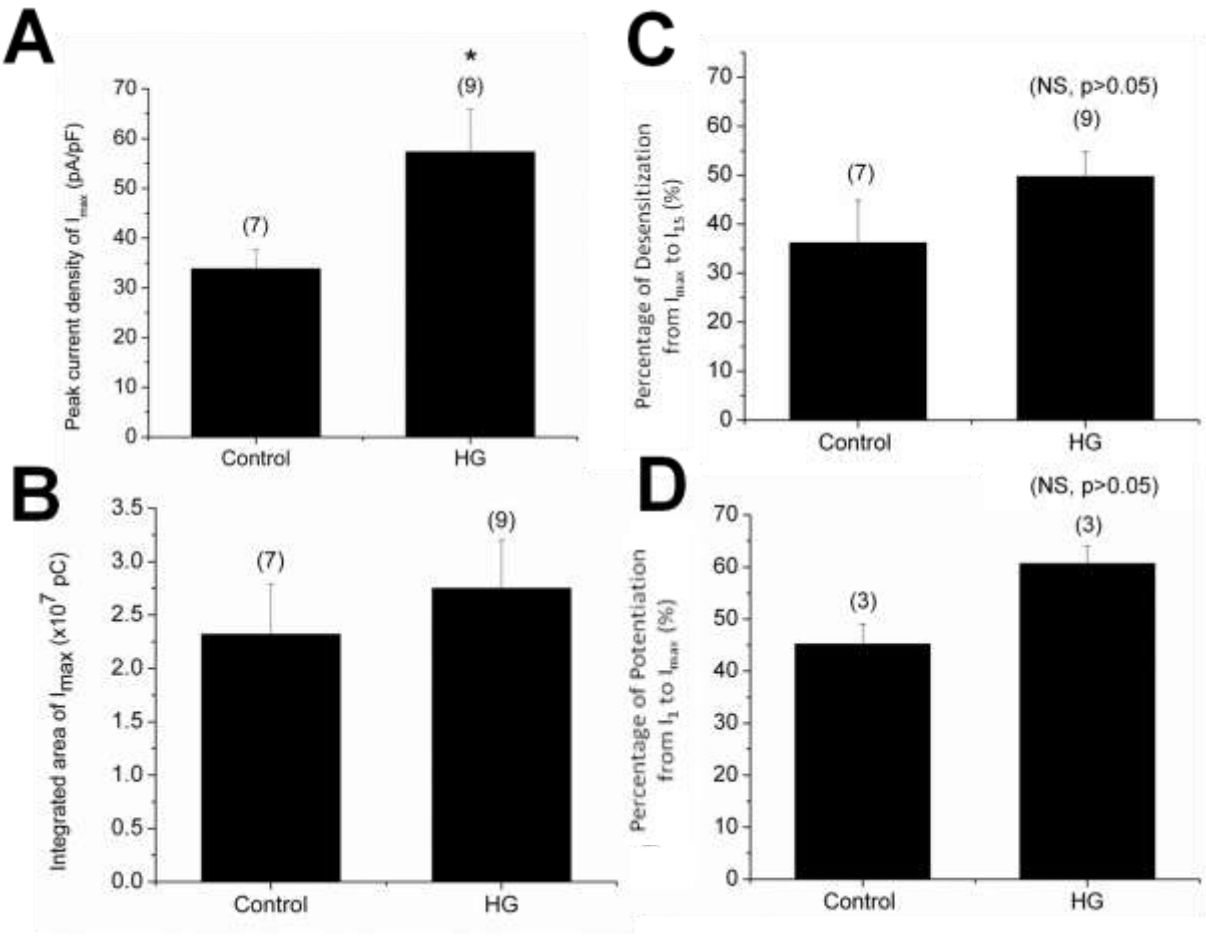

Figure 3-8. **Adult DRG neurons also show enhancement in TRPV1-mediated current for peak current density but not integrated area.** Bar graphs summarizes the mean± SEM current density **(A)** and integrated area **(B)** of the I_{max} CAP-evoked current for neurons treated in control and HG conditions. *p<0.05. Bar graphs (**C** and **D**) summaries the mean± SEM percentage of desensitization and potentiation in TRPV1-mediated currents from control and high glucose conditions with respect to the monophasic and biphasic responses.

3.4 Discussion

Although glucose-mediated mechanisms leading to oxidative stress in sensory neurons as a result of hyperglycemia have been demonstrated to contribute to the pathogenesis of diabetic sensory neuropathy, very little is known of the cellular and molecular mechanisms influenced by elevated cytoplasmic ROS and its effects on the functional activity of the neuron. In the present study, I demonstrated that a high glucose insult of 25 mM for 7 days generated high levels of cytoplasmic ROS in neonatal DRG neurons compared to basal glucose levels of 5 mM in the control condition. This in turn enhanced the CAP-evoked current in both monophasic and biphasic responses, while having no influence on P2X-mediated currents. Furthermore, I have shown that the mechanisms driving desensitization or potentiation in the monophasic and/or biphasic responses of the TRPV1 receptor were unaffected under high glucose conditions. However, the enhancement of TRPV1-mediated currents was reversed when cytoplasmic ROS levels were reduced with an antioxidant mixture containing α-lipoic acid and catalase. Collectively, these findings demonstrated the influential role of hyperglycemia-induced oxidative stress in facilitating TRPV1 activity and its implications on the onset of DSN. More importantly, I show that the redox state of the sensory neuron, in particular, can influence the functional activity of TRPV1 receptors.

3.4.1 P2X receptors are unaffected by high glucose-induced oxidative stress

Evidence from genetic and pharmacological approaches in the deletion or down regulation of P2X3 receptors revealed the importance of this receptor subtype in pain processing involved in thermal and mechanical nociception (reviewed by Jarvis, 2003). Information on the influence of hyperglycemia- induced oxidative stress on the functional activity of this receptor subtype remains limited in literature. In the current study, I demonstrated that repeated application of ATP evoked a fast-inactivated current resembling the kinetics of the P2X3 receptor subtype (Grubb & Evans, 1999), confirming that this subtype is the dominant P2X receptor in small to medium size DRG neurons. While the findings demonstrated no change in the P2X-mediated currents following seven days of the high glucose insult, this suggested that the P2X receptor was not affected under oxidative stress. It is possible that long-term exposure of high glucose may be required to see an effect as demonstrated in streptozotocin (STZ) models of diabetes, which revealed an increased expression and function of P2X2 and/or P2X3 receptors

that paralleled the onset of hyperalgesia in diabetic mice 2 weeks after STZ injections (Migita et al., 2009) and rats (Gy, Li, Liu & Huang (2011). Whether prolonged exposure to the high glucose insult (i.e. greater than 7 days) influences the functional activity of P2X receptors in sensory neurons *in vitro* may need to be evaluated.

3.4.2 High glucose enhanced TRPV1-mediated currents

The influence of hyperglycemia on TRPV1 receptors expressed in sensory neurons have been studied in animal models with long standing diabetes induced by STZ (Pabbidi et al., 2008 a; Bishnoi et al., 2011). A common observation in these studies is that a small number of STZ-induced animals were not hyperglycemic but displayed hyperalgesia upon behavioural testing. Pabbidi et al (2008b) had demonstrated that DRG neurons in culture exposed to STZ generated high levels of ROS, and was associated with an increase in TRPV1 activity and expression independent on the glycemic state, suggesting that STZ is a confound variable to consider.

In our *in vitro* model, I investigated the effect of high glucose on TRPV1-mediated currents. In the current study, the effects of high glucose on these currents (i.e. monophasic and biphasic responses), which were characterized in Chapter 2, were examined. Interestingly, the two types of responses persisted in the high glucose condition, suggesting that these responses are an inherent property of TRPV1 receptors occurring independently of treatment conditions. It was interesting to find that the mechanisms of desensitization or potentation observed in the monophasic and/or biphasic responses of TRPV1 behaviour were unaffected under the high glucose condition, but that quantitative analysis of the current influx revealed a robust enhancement for both the peak current density and integrated area under high glucose. This suggests that in the high glucose condition cells are more responsive to pain stimulation, such that a greater magnitude of membrane current is observed in TRPV1 receptors. Similarly, this enhancement of the current was also reported in DRG neurons of STZ-induced diabetic rats revealing an increase in peak amplitude and the duration of current influx (Hong & Wiley, 2005). In contrast, oxidative modulation of TRPV1 receptor was reported to slowly sensitize TRPV1-mediated currents in the presence of hydrogen peroxide in HEK cells through covalent modification of cysteine residues forming inter-cysteine disulfide bonds in the cytoplasmic termini of the receptor (Chuang & Lin, 2009). Although this study did not show desensitization in TRPV1-mediated currents, the authors postulated that the potentiated TRPV1

receptor may not desensitize. Conversely, the present study demonstrated that high glucose enhanced the CAP-evoked current, which was mediated by cytoplasmic ROS, but had no influence on the type of response (i.e. monophasic and biphasic) and its desensitization.

In addition to characterizing this robust enhancement in the monophasic and biphasic responses, I further show that the increase in CAP-evoked current was strongly driven by calcium influx. This revealed that the calcium influx affected the type of responses to CAP and the desensitization as described in Chapter 2. Here, I demonstrated that calcium was essential for the enhancement of CAP-evoked currents in the high glucose condition. Because the influx of current in the barium experiment reflected barium influx as well as other monovalent ions (Comunanza et al., 2011), quantitative analysis of the difference in calcium influx without the inclusion of the barium current could not be done. Interestingly, a common observation in type 1 and type 2 animal models of long standing diabetes is the high resting intracellular calcium concentrations in DRG neurons (reviewed by Fernyhough & Calcutt, 2011). Whether the calcium-mediated potentiation of TRPV1 instigates this calcium abnormality phenomenon remains to be explored.

3.4.3 Antioxidants reversed the enhancement of TRPV1-mediated currents

The approach of using antioxidants to prevent oxidative stress and the progression of DSN has shown to be a therapeutic avenue for promoting neuronal protection and survival (Vincent et al., 2005b; Vincent et al., 2009). However, denotation of the influence of antioxidants on the functional activity of sensory neurons has been limited in the literature. In the present study, I have demonstrated that elevation of cytoplasmic ROS, induced by the high glucose insult in DRG neurons in TRPV1 current. This effect was reversed by antioxidants α-lipoic acid and catalase in the recording pipette. While antioxidants might be a useful strategy to alleviate oxidative stress and the enhancement in TRPV1-mediated currents, as demonstrated in the present study, it should be acknowledged that I observed a change in the behaviour of TRPV1-mediated currents in both control and high glucose neurons treated with the antioxidants. This suggests that the redox state of the neuron influences the sensitivity of TRPV1 receptors prior to exposure to a stimulus. It is possible that ROS-mediated oxidation also influenced other substrates and their activity within the cytoplasm. Mild oxidation in neurons has been shown to mediate thiol oxidation in PKC and increase the kinases' activity (Knapp & Klann, 2000). In

examining the influence of PKC activity in the biphasic response reported in Chapter 2, I demonstrated that application of a PKCβ inhibitor drastically enhanced the degree of desensitization of the monophasic response, in which currents displayed an initial large inward current followed by a second inward current that was half the size and gradually desensitized following repeated applications of CAP. This current response resembled the current responses obtained in the present study when high glucose and control (trace not shown) neurons were treated with antioxidants. It is possible that the presence of antioxidants can reduce PKC activity and subsequently alter the competition between kinases and phosphatases (i.e. calcineurin) that would normally influence the potentiation or desensitization of TRPV1 receptors upon CAP applications. Because intracellular application of antioxidants only evoked one type of response in TRPV1 function from a small sample of high glucose neurons, it remains unknown whether this reduced effect abolished both the monophasic and biphasic responses or if increasing the sample size would reveal two distinct responses, similar to the findings in the PKCβ experiment and consistent with the observations that there are two functionally distinct populations of neurons. Collectively, these findings suggest that the redox state of the sensory neuron can influence TRPV1 activity, and possibly the distribution of the monophasic and biphasic responses observed. Certainly, the mechanisms and implications of these current responses will need to be addressed in future studies.

3.4.4 Age-related vulnerability to oxidative stress and its influence on TRPV1- mediated currents

It has recently been shown that TRPV1 receptor expression is maintained post development in peptidergic neurons, while down-regulated in nonpeptidergic neurons (Cavanaugh et al., 2011). To show that ROS-mediated enhancement in TRPV1 receptors occurred regardless of development, transgenic mice that express the fluorescent marker GFP in neurons that express the CGRP promoter was used to selectively identify CGRP-positive peptidergic neurons in culture. Consistent with neonates, repeated applications of CAP revealed the monophasic and biphasic responses in both control and high glucose conditions.

The influence of the high glucose insult on adult DRG neurons revealed a different enhancement in TRPV1-mediated currents. In the present study, it was observed that this effect was largely attributed to the peak amplitude but had no effect on the integrated area in adult

DRG neurons. However, due to the low sample size, analysis was conducted to compare control and high glucose conditions irrespective of the type of response. The differential effect in adults may be attributed to this factor, which makes it difficult to draw definitive conclusions. Increasing sample size will help study the difference between neonates and adults.

3.5 Conclusion

In summary, the results provide evidence that elevation of cytoplasmic ROS in DRG neurons as a result of high glucose causes the enhancement of TRPV1 currents following repeated applications of capsaicin, while having no influence on P2X receptors. This effect on TRPV1 was reversed when cells were exposed to an intracellular antioxidant mixture. This specific oxidation of the TRPV1 channel and not P2X receptor constitutes a mechanism leading to an increase in the functional activity of sensory neurons and in the onset of diabetic sensory neuropathy.

CHAPTER 4
GENERAL DISCUSSION

4.1. Summary of main findings

In the present thesis, two studies were conducted to examine the influence of high glucose on P2X and TRPV1 receptors in dissociated lumbar DRG neurons in culture. While investigating the role of TRPV1, two types of responses were observed following repetitive CAP applications under the control condition: (1) a 'monophasic response' with a diminuation of peak current typical of tachyphylactic desensitization; or (2), a 'biphasic response' composed of an initial gradual potentiation of the peak current followed by its desensitization by tachyphylaxis. The latter response was a novel observation that prompted further investigations in Chapter 2. I demonstrated that the initial CAP-evoked current could help identify the monophasic and biphasic responses. In addition, it was observed that the degree of desensitization was similar between responses, which suggested a common desensitization mechanism. Furthermore, inhibition of PKCβ, a PKC isoform that has been implicated in diabetic hyperalgesia, prevented the potentiation that was characteristic of the biphasic response, suggesting that this isoform of PKC mediated the potentiation phenomenon in the biphasic response.

Following the characterization of the two types of responses to repetitive CAP applications under control conditions, the influence of oxidative stress during high glucose on the monophasic and biphasic responses of TRPV1 receptors were examined in Chapter 3. Both TRPV1 responses were enhanced under high glucose conditions without affecting the desensitization and/or potentiation in the monophasic or biphasic response. This suggested that the TRPV1 receptor may play a role in mechanisms of diabetic sensory neuropathy. In contrast, P2X-mediated currents remained unaffected by the high glucose condition. It is possible that a longer period of high glucose is required to see the effects on P2X receptor that has been reported in animal models of long-standing diabetes (Migita et al., 2009; Gy et al., 2011).

To further explore the enhanced effect in TRPV1-mediated currents under high glucose, additional experiments were conducted. I demonstrated that this enhanced phenomenon was

greatly dependent on calcium influx. Furthermore, antioxidant treatment in neurons that have been exposed to high glucose for a week reversed the enhancement in current influx; however, a secondary effect was observed in that these currents were neither monophasic nor biphasic in response. This suggested that the redox state of the cell may influence the type of responses (i.e. monophasic or biphasic) in TRPV1 receptors. Lastly, I also demonstrated that adult nociceptive neurons also exhibit the monophasic and biphasic responses in both control and high glucose conditions. Despite the small sample size, these cells displayed a differential effect on the enhancement of CAP-evoked currents that was not as robust as neonates. This suggested that the difference in the effect of oxidative stress on TRPV1 receptors may be attributed to age-related vulnerability and the antioxidant capacity as it was previously described for central neurons (Ferriero, 2001; Aycicek & Iscan, 2006; Perrone et al., 2010).

Collectively, these experiments revealed that the intrinsic properties of the cell (i.e. calcium-dependent endogenous activators, and the redox state) can influence the monophasic and biphasic responses of TRPV1 receptors under control and diabetic conditions. In addition, I demonstrated that the TRPV1 receptors are affected by the high glucose insult as early as 7 days, suggesting that this receptor could be involved in the onset of diabetic sensory neuropathy.

4.2 Intrinsic properties of the sensory neuron that can influence the function of TRPV1 receptors

4.2.1 Biphasic response of TRPV1 receptor is regulated by phosphorylation of PKCβ

In Chapter 2, the unexpected observation that TRPV1 receptors can respond in two ways (i.e. monophasic and biphasic) following repetitive applications of CAP was a novel finding that prompted further investigations to characterize these responses before examining the receptor under the high glucose condition. Several hypotheses were introduced to explain the mechanism regulating the monophasic and biphasic response of TRPV1 receptors under the control condition, independent of inflammatory mediators. It was proposed that the type of response was regulated by the phosphorylated or dephosphorylated state of the receptor prior to CAP applications (refer to 2.4.2). Based on the PKCβ experiment, it appeared that the potentiated phenomenon in the biphasic response was mediated by PKCβ activity, since

inhibition of this kinase abolished the potentiation phenomenon. Similarly, inhibition of PKCβ revealed a robust desensitization phase in the monophasic response suggesting that a basal level of PKCβ activity is required for the gradual desensitization observed in the monophasic response. Furthermore, it would be interesting to understand the implications of the monophasic and biphasic response in the presence of other noxious stimulus (i.e. pH, proinflammatory mediators, temperature and etc) that regulate TRPV1 receptors. Thus, providing a greater understanding of how these responses would contribute in an animal that is feeling pain. For example, does the biphasic response act as a backup sensory system to allow for the detection of potential nociceptive stimuli when neurons displaying the monophasic response have already been desensitized?

4.2.2 Effect of cytoplasmic ROS on TRPV1 receptors

In recent studies, acute exposures to mild oxidants have shown to influence the sensitivity of the TRPV1 receptors expressed in HEK cells, increasing the membrane current following repeated exposure to the receptor's agonist (Susankova et al., 2006; Chuang & Lin, 2009). This suggested that oxidative stress may also regulate TRPV1 receptors in pain processing during inflammation, inflection and tissue injury, all conditions associated to oxidative stress. However, the influence of mild oxidants or oxidative stress on native TRPV1 receptors in DRG neurons has not been examined to date. In the present thesis, elevated ROS as a result of prolonged exposure to high glucose revealed three things: 1) oxidative stress influenced the magnitude of CAP-evoked current through the receptor; 2) oxidative stress may alter PKCβ activity and subsequently affect TRPV1 receptors; and 3) age-related vulnerability to oxidative stress influenced the degree of enhancement in membrane current during high glucose.

4.2.2.1 Oxidative stress influences the magnitude of current through TRPV1 receptors

In the present thesis, it was demonstrated that high glucose-induced oxidative stress enhanced the calcium-dependent membrane current generated from repeated applications of CAP without affecting the monophasic or biphasic response in TRPV1 receptors. Because the parameters of the monophasic and biphasic responses in native TRPV1 receptors remained

unaffected by high glucose, this contrasted with recent reports that demonstrated that TRPV1 receptors overexpressed in HEK cells and exposed to ROS always displayed a gradual potentiation in the membrane current resembling the biphasic response (Susankova et al., 2006; Chuang & Lin, 2009). It is possible to consider that the length of time the sensory neuron is exposed to the mild oxidant could influence the type of potentiation in TRPV1 receptors following repetitive activation. While the aforementioned studies exposed cells to approximately 2 minutes of the mild oxidant, the present thesis examined sustained oxidative stress conditions (induced by 7 days in high glucose) in the *in vitro* diabetes model (Vincent et al, 2005). Nevertheless, it is plausible to suggest that acute or long-term oxidative stress have a differential effect on TRPV1 function. However, the mechanism that triggers the enhancement of current observed during high glucose-induced oxidation in the present thesis remains poorly understood.

Recently, it was revealed that as in other calcium-permeable channels (e.g. TRPA1 and P2X receptors, Banke et al., 2010 & 2011; Virginio et al., 1999) pore dilation plays a role in TRPV1 receptors. Repetitive CAP application led to the calcium-dependent pore dilation of the TRPV1 receptors, permeating large fluorescent molecules (i.e.Yo-PRO-1) in cells (Bautista & Julius, 2008; Chen et al., 2009; Li et al., 2011). It is possible to consider that the mechanism for pore dilation is enhanced for cells exposed to the high glucose insult, thereby allowing a greater volume of charge through the ion channel following repeated applications of CAP. Although, the mechanisms that regulate pore dilation of TRPV1 receptors remain unclear, pathological conditions could contribute to this effect, sensitizing sensory neurons to noxious stimuli. Here, I show that reducing cytoplasmic ROS levels prevented the enhancement of current influx in high glucose conditions. It would be interesting to investigate in future experiments whether pathological signals linked to high glucose could trigger pore dilation of TRPV1 and how that plays a role in the context of diabetes.

4.2.2.2 Oxidative stress may modulate TRPV1 receptors through PKCβ activity

An interesting observation was found in the PKCβ study in which cells that exhibited the monophasic response under the influence of the PKCβ inhibitor were comparable in their responses to DRG neurons exposed to antioxidants. It has been reported that ROS can increase PKC activity through thiol oxidation (Knapp & Klann, 2000; Lin & Takemoto, 2005). Indeed, it is possible that oxidative stress and the generation of ROS can not only influence membrane

receptors (i.e. TRPV1) but also endogenous activators (i.e. PKC) that regulate them, leading to amplification in receptor activity. Based on the present thesis it remains unknown whether the current traces generated in the presence of the antioxidants were due to isolated or a combination of events involving reducing agents targeting the TRPV1 receptor and/or PKC activity.

4.2.2.3 Age-related vulnerability to oxidative stress influences the degree of enhancement in membrane current

Although the mechanism by which oxidative stress enhanced the membrane currents of TRPV1 remains unclear, my data indicates that the degree of enhancement was dependent on the age of the DRG neurons in culture, suggesting age-related vulnerability to oxidative stress. Studies investigating cerebral ischemia, epilepsy, and mitochondrial disorders in newborns have suggested a reduced antioxidant capacity to regulate oxidative stress in central neurons (Ferriero, 2001; Aycicek & Iscan, 2006; Perrone et al., 2010). Under the high glucose insult, a greater degree of enhancement was observed for neonatal DRG neurons relative adult DRG neurons. It is possible that neonatal DRG neurons under the high glucose insult greatly diminished its antioxidant capacity within 7 days of treatment, while adult DRG neurons retained some of its antioxidant capacity to reduce oxidative stress. In addition, it would suggest that a longer exposure to high glucose in adult DRG neurons may show reproducible results similar to neonatal DRG neurons observed in the present thesis, as well as studies that have examined animal models of long-term diabetes.

4.3 Future Directions

4.3.1 Calcium imaging to parallel electrophysiological recordings in understanding the role of TRPV1 in physiological and high glucose conditions

A number of electrophysiological studies dating back to the late 1980s have identified the influential role of calcium in regulating TRPV1 activity (Wood et al., 1988; Petersen et al., 1996; Oh et al., 1996; Koplas et al., 1997). In Chapter 2, based on the characterization of the monophasic and biphasic responses it cannot be excluded that intracellular calcium may

influence TRPV1 function playing a role in the generation of these two responses. To study whether intracellular calcium contributes to the monophasic and biphasic responses we plan to use time-lapse calcium imaging to measure the spatial and temporal profiles of induced changes in the intracellular calcium concentrations. The combining of this data with my electrophysiological findings would shed light on the influential role of intracellular calcium on the behaviours of TRPV1 receptors. Certainly, previous studies have used this method to measure changes in intracellular calcium concentrations as a result of activating TRPV1 in the presence of pharmacological manipulations on the receptor (Honan & McNaughton, 2007; Sculptoreanu, Kullmann & de Groat, 2008; Zhang et al., 2010).

Moreover, in Chapter 3, it was demonstrated that there was an enhancement in TRPV1-mediated currents that was calcium dependent under the influence of high glucose, based on the observation made from the barium experiment. The use of this technique can further address or explore issues generated from the current thesis: 1) Is a high glucose insult of at least 7 days capable of inducing a dysfunction in intracellular calcium homeostasis as reported in long standing diabetes model of type 1 and type 2 (Fernyhough & Calcutt, 2010); and 2) Is the enhanced effect of TRPV1 receptor influenced by the dysfunction of intracellular calcium homeostasis or is the effect solely mediated by ROS-oxidation of TRPV1 receptors.

In summary, this thesis presented novel insights in the role of high glucose-induced oxidative stress on the functional modification of TRPV1 receptors in sensory neurons. Here, I show that TRPV1 receptors exhibited two types of responses in the control condition (Figure 4-1A). For the monophasic response, application of CAP changes the conformation of the TRPV1 channel from a closed to an open state allowing the influx of current. As previously described, entry of calcium or calcium dependent phosphatase mediates the desensitization phenomenon displayed by the run-down in peak current (Docherty et al., 1996; Koplas et al., 1997). Similarly, for cells that responded in a biphasic response, application of CAP opened the channel allowing the influx of current, however, I demonstrated that the potentiated phenomenon was mediated by PKCβ. Under high glucose treatment, which resulted in the elevation of cytoplasmic ROS, I demonstrated that the magnitude of current generated through TRPV1 channel was enhanced following a series of CAP applications for both the monophasic and biphasic responses (Fig. 4-1B). But had no influence on the mechanism that mediated the desensitization or potentiation that was characteristic of the responses. Furthermore, I have shown that reducing

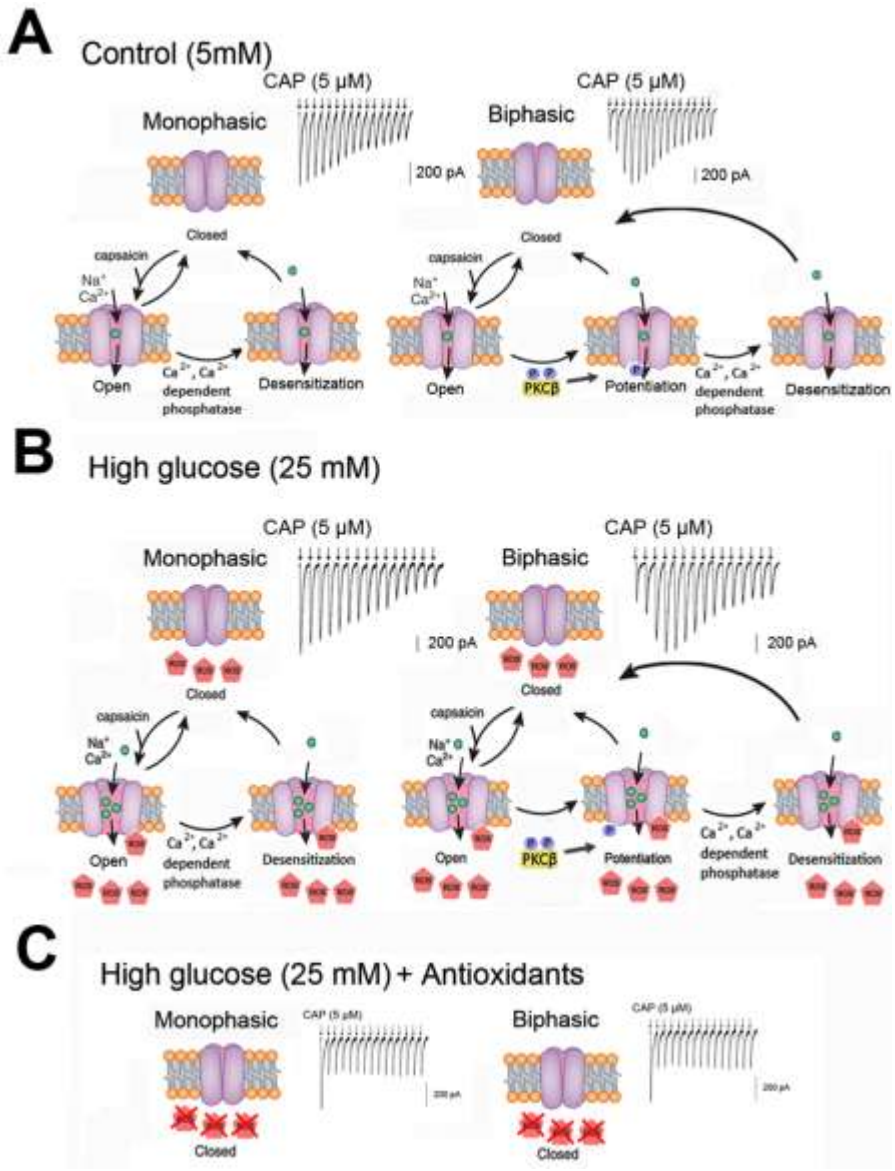

Figure 4-1. Proposed working models for the funcational states of TRPV1 receptors under control and high glucose conditions. (A) A series of CAP applications evoked two types of responses in TRPV1 activity: a monophasic (left) and biphasic (right) response, which is represented by the current traces. For the monophasic response, application of CAP changes the conformation of the channel from a closed to an open state allowing the influx of current, where entry of calcium or calcium dependent phosphatase mediates the desensitization phenomenon. Similarly, for cells that responded in a biphasic response, application of CAP opened the channel allowing the influx of current, however, PKCβ mediated the potentiation phenomenon. **(B)** Under high glucose treatment, which resulted in the elevation of cytoplasmic ROS, the magnitude of current influx was enhanced for both the monophasic and biphasic responses. But had no influence on the mechanism that mediated the desensitization or potentiation that was characteristic of the responses. **(C)** While reducing ROS reversed this enhancement as a result of high glucose, however, the sensitivity of TRPV1 channels as the current responses was neither monophasic nor biphasic.

cytoplasmic ROS reversed this enhancement as a result of high glucose, however, affected the sensitivity of TRPV1 channels as the current responses was neither monophasic nor biphasic (Fig.4-1C). These findings suggest that the redox state of the cell influences TRPV1 activity and collectively correlate with the pathological environment reported in diabetes, and therefore may have implications in pain perception and processing in the diabetic state.

CHAPTER 5
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